Investigation into the effects of specific muscarinic acetylcholine receptor antagonists on the myocardium in pre-clinical conditions of ischaemia reperfusion injury and oxidative stress model

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Award date:
2015

Awarding institution:
Coventry University

Investigation into the effects of specific muscarinic acetylcholine receptor antagonists on the myocardium in pre-clinical conditions of ischaemia reperfusion injury and oxidative stress model

By

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November 2015

A thesis submitted in partial fulfilment of the University's requirements for the Degree of Doctor of Philosophy

Acknowledgements

I would like to express my deepest appreciation to my supervisors; Prof. Helen Maddock and Dr. Afthab Hussain for giving me the opportunity to undertake this project and also for their continuous support throughout my PhD. I would also like to thank Coventry University and Brookes Pharmaceuticals for funding my PhD. In addition, I would also like to thank the lab technician, Mark Bodycote for his support and patience throughout the period of my PhD.

Furthermore, I would like to express my gratitude to my friends and lab colleagues Aaron Nagra, Maryam Babba and Mayel Gharanea for their support and friendship.

Most importantly, I am indebted to my parents, my wife and her parents for their continuous encouragement, love and support at all times throughout my PhD. I am very privileged to have such a loving and caring family around me as without their assistance, completing my PhD would not have been possible.

Abstract

Muscarinic acetylcholine receptors (mAChRs) are G-protein coupled receptors that mediate various actions of Acetylcholine (ACh) in the central nervous system and peripheral nervous system. In mammals, five distinct mAChR subtypes (M_1-M_5) have been recognised with the M_2 subtype being predominantly present in the heart. The mAChR antagonists are routinely used for the treatment of various pathophysiological conditions including respiratory conditions. However, it has been postulated that mAChR antagonists may increase morbidity and mortality in chronic obstructive pulmonary disorder (COPD) and asthma patients with underlying cardiovascular disease, raising concerns regarding the cardiovascular safety of these agents. The current study was therefore undertaken to investigate the effects of individual mAChR antagonists in the setting of myocardial ischaemia reperfusion injury and oxidative stress models. We also investigated whether the inhibition of the mitochondrial permeability transition pore (MPTP) with cyclosporine-A (CsA) in the presence and absence of individual mAChR antagonists provided protection against ischaemia reperfusion injury. Furthermore, we also aimed to investigate the intracellular signalling pathway associated with mAChRs antagonists mediated myocardial injury under the stress conditions.

Langendorff results showed that the non-selective M_1-M_3 mAChR antagonist, ipratropium bromide, the M_2 mAChR antagonist, AF-DX 116 and the M_3 mAChR antagonist, DAU 5884 significantly increased the infarct size to risk ratio of the heart in conditions of ischaemia and reperfusion. Detrimental effects of AF-DX 116 and DAU 5884 were abrogated by co-treatment of these drugs with mAChR agonist, acetylcholine (ACh) and/or CsA. Cell viability data of isolated cardiac myocytes revealed that AF-DX 116 and DAU 5884 caused a concentration

dependent decrease in the viability of cardiac myocytes as well as causing a reduction in the time taken to depolarisation and hypercontracture under oxidative stress. AF-DX 116 and DAU 5884 significantly increased the levels of p-SAPK/JNK and decreased the levels of p-Akt and p-ERK. In addition, ACh and CsA showed to activate p-Akt and p-ERK.

To conclude, the data suggest that AF-DX 116 and DAU 5884 caused cardiotoxicity at cellular, tissue and protein level in conditions of ischaemia reperfusion injury and oxidative stress. Furthermore, inhibition of the mitochondrial transition pore with CsA protected against the AF-DX 116 and DAU 5884 induced injury via activation of the pro-survival proteins, p-Akt and p-ERK.

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Abbreviations

%	**Percentage**
µM	**Micromole**
AC	**Adenylyl cyclase**
ACh	**Acetylcholine**
AChE	**Acetylcholinesterase AChE**
AF-DX 116	**11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one**
AMI	**Acute myocardial infarction**
AMP	**Adenosine monophosphate**
Apaf-1	**Adaptor protease activating factor-1**
AIF	**Apoptosis inducing factor**
Akt	**Protein Kinase B**
ANT	**Adenine nucleotide translocase**
ATP	**Adenosine triphosphate**
BNP	**B-type natriuretic peptide**
cAMP	**Cyclic adenosine monophosphate**
ChAT	**Choline acetyl transferase**
CF	**Coronary flow**
cGMP	**Cyclic guanosine monophosphate**
CsA	**Cyclosporine A**
CoA	**Coenzyme A**
COPD	**Chronic obstructive pulmonary disease**
CHD	**Coronary heart disease**

CK-MB	**Creatine kinase isoenzyme MB**
cTnI	**Cardiac troponin I**
cTnT	**Cardiac troponin T**
CVD	**Cardiovascular disease**
Cyp-D	**Cyclophilin D**
DAG	**Diacylglycerol**
DAU 5884	**8-Methyl-8-azabicyclo-3-endo[3.2.1]oct-3-yl-1,4-dihydro-2-oxo-3(2H)-quinazolinecarboxylic acid ester hydrochloride**
DISC	**Death inducing signalling complex**
DMSO	**Di-methyl sulfoxide**
ECG	**Electrocardiogram**
EGFR	**Epidermal growth factor receptor**
eNOS	**Endothelial nitric oxide synthase**
ERK 1/2	**Extracellular signal-regulated kinase 1 and 2**
IBS	**Irritable bowel syndrome**
FasL	**Fas ligand**
FEV1	**Forced expiratory volume**
GDP	**Guanosine diphosphate**
GTP	**Guanosine triphosphate**
GEFs	**Guanine nucleotide exchange factors**
GPCR	**G-protein coupled receptors**
HEPES	**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
HeNe	**Helium-Neon**
HR	**Heart rate**
IHD	**Ischaemic heart disease**

Ipratropium	**3-(3-hydroxy-1-oxo-2-phenylpropoxy)-8-methyl-8-(1-methylethyl)-, bromide**
IR	**Ischaemia reperfusion**
JNK	**C-Jun N-terminal kinase**
KHB	**Krebs Henseleit Buffer**
LDL	**Low density lipoprotein**
LVDP	**Left ventricular diastolic pressure**
mAChR	**Muscarinic acetylcholine receptor**
MAPK	**Mitogen activated protein kinase**
miRNAs	**MicroRNAs**
MMP9	**Matrix metalloproteinase-9**
MPTP	**Mitochondrial permeability transition pore**
mRNA	**Messenger RNA**
mTOR	**Mammalian target of rapamycin**
MTT	**(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)**
NADPH	**Nicotinamide adenine dinucleotide phosphate**
NHE	**Na+/H+ exchanger**
NMDA	**N-Methyl-D-aspartate**
O2-	**Superoxide anion**
OAB	**Overactive bladder**
PDE	**Phosphodiesterase**
PCR	**Polymerase chain reaction**
PDGFR	**Platelet-derived growth factor receptor**
PI3K	**Phosphoinositide 3-kinase**
PIP_2	**Phosphatidylinositol 4, 5-biphosphate**
PIP_3	**Phosphatidylinositol 3, 4, 5-triphosphate**

PKC	**Protein kinase c**
PWV	**Pulse wave velocity**
PVDF	**Hybond-P Polyvinyl Difluoride**
RISK	**Reperfusion injury salvage kinase**
ROS	**Reactive oxygen species**
SMAC	**Second mitochondria-derived activator of caspases**
SAPK	**Stress activated protein kinase**
TBS	**Tris-buffered saline**
Telenzepine	**4,9-Dihydro-3-methyl-4-[(4-methyl-1-piperazinyl)acetyl]-10H-thieno[3,4-b][1,5]benzodiazepin-10-one dihydrochloride**
TMRM	**Tetramethylrhodamine methyl ester**
TNF	**Tumour necrosis factor**
TSPO	**Translocator protein**
TTC	**Triphenyltetrazolium chloride**
VDAC	**Voltage dependent anion channel**

Chapter One: General Introduction

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is characterised by chronic inflammation in the airways and lung parenchyma (Huiart *et al*. 2005). It is a term for a collection of airway diseases such as chronic bronchitis, emphysema and chronic obstructive airways disease. Its clinical symptoms include shortness of breath, excessive coughing, sputum production and frequent chest infections (Vestbo and Lange 2014).

In addition to the airflow obstruction, there is increasing evidence that COPD is also responsible for other systemic pathologies including cardiovascular diseases (CVD) (Macnee *et al*. 2008, Maclay *et al*. 2007). The common risk factors for both the conditions include smoking, sedentary lifestyle, advanced age and poor diet (Maclay *et al*. 2007). Huiart *et al*. (2005) showed in a study that among COPD patients, CVD was an important cause of hospitalisation and death. The occurrence of hospitalisation for CVD was higher than that for COPD itself. Although the exact mechanism for the increase in risk of CVD in COPD patients is not entirely understood, there are proposed hypotheses about systemic inflammation being involved. Systemic inflammation is caused as a result of chronic activation of innate immune system and excessive release of pro-inflammatory cytokines such as interleukins and tumour necrosis factor from immune cells including macrophages, monocytes, B lymphocytes, T lymphocytes and mast cells (Lacy and Stow 2011).

An increase in systemic inflammatory response has been linked with acute cardiovascular events as it is potentially involved in atherosclerosis development (Clayton *et al*. 2008). Studies have also shown that there is an increase in systemic inflammation in COPD patients activating circulating leukocytes (Wouters 2005). The direct "spillover" of lung inflammation to the

systemic circulation caused by hyperinflation of lungs, tissue hypoxia, muscle dysfunction and bone marrow stimulation is a possible reason for the origin of the systemic inflammatory response in COPD patients (Macnee *et al*. 2008). In addition, activated peripheral blood neutrophils release reactive oxygen species (ROS), have increased expression of adhesive molecules and extracellular proteolysis. These are common factors in atherosclerosis pathogenesis and are also observed in COPD patients. In addition, it has been reported that in COPD patients circulating monocytes release increased levels of matrix metalloproteinase-9 (MMP9) which is also involved in atherosclerosis progression and plaque rupture (Aldonyte *et al*. 2003). Changes in arterial stiffness have also been considered as a potential mechanism to link between COPD and CVD. Central arterial stiffness has been shown to predict cardiovascular mortality, and is calculated using aortic pulse wave velocity (PWV), and an increased arterial stiffness has also been found in COPD patients (Sabit *et al*. 2007).

COPD patients are subjected to sustained hypoxia such as in a severe disease state, respiratory failure or intermittent hypoxia such as during exacerbations and exercise (Macnee *et al*. 2008). Hypoxia has been shown to increase systemic inflammation, oxidative stress, cause up-regulation of adhesive cell molecules and induce hemodynamic stress, factors that influence atherogenesis (Lattimore *et al*. 2005, Ichikawa *et al*. 1997). Acute hypoxia has also been shown to induce hemodynamic stress increasing heart rate and cardiac index (Thomson *et al*. 2006). These effects could be relevant for COPD patients who are subjected to acute and intermittent hypoxia during exacerbations and exertion.

1.1.2 COPD treatment

Although there is no definite cure for COPD, treatment mainly aims to treat the symptoms, reduce the risk factors, manage COPD and associated illnesses and finally delay its progression (Rabe *et al*. 2007, Vestbo and Lange 2014). The most important measures that have been shown to reduce COPD mortality are cigarette smoking cessation and providing supplemental oxygen to the patients (Drummond et al. 2008). Studies have shown that COPD mortality can be reduced by 18% by cigarette smoking cessation (Decramer *et al*. 2012).

Inhaled bronchodilator therapy is the most important medication used in the management of COPD (Decramer *et al*. 2012). The two main types of bronchodilators are β_2 agonists and anticholinergics. Both these types exist in short-acting and long-acting forms and are used to reduce the exacerbations associated with COPD.

As COPD is characterised by airway inflammation and bronchoconstriction, there are increased levels of ACh in the respiratory tract, and the vagal tone is believed to be the single reversible component, anticholinergics constitute a reasonable bronchodilator therapy. Various studies have shown an improved function and exercise tolerance in COPD patients being treated with anticholinergics (Crockett 2000, Halpin 2001). Anticholinergics block the cholinergic tone and relax the smooth muscle in the airways. Anticholinergics have shown to have a greater response in COPD patients than β_2 agonists (Barnes 1999, Cooper and Tashkin 2005).

The medicinal smoke of burning roots and stems of the *Solanaceae* plants releases an aerosol of potent alkaloids such as atropine. For many centuries, inhalation of this smoke has been used to treat respiratory disorders worldwide (Chapman *et al*. 2006). It was in the early 18th century that atropine was first used to treat asthma in Britain in the form of a pipe tobacco (Gandevia 1975). Since then, atropine based agents have been commonly used to treat respiratory disorders.

However, due to its poor absorption across the oral mucosa and via the gastrointestinal tract and the adverse side effects associated with it including; dry mouth, dizziness, hallucinations, nausea and tachycardia led to a decline in its usage in 1930's (Chapman *et al*. 2006).

Following studies recognised that the inhibition of cholinergic system could provide an alternative therapeutic approach to improve airflow thus reviving an interest in the use of anticholinergics (Gandevia 1975, Barnes 1986). The airway smooth muscle consists of M_2 and M_3 muscarinic acetylcholine receptor (mAChR) subtypes but the latter are mainly involved in bronchial and tracheal smooth muscle contraction (Van Nieuwstadt et al. 1997). This is shown in diverse species, including humans, by the functional affinities of various subtype selective antagonists (Roffel et al. 1990, Ten Berge et al. 1993). However, a minor role of M_2 subtype has also been shown to mediate airway smooth muscle contraction in peripheral airways (Struckmann et al. 2003).

Increased release of ACh and abnormal mAChR expression either via increased levels of M_1 and M_3 subtypes, or M_2 subtype dysfunction by $C8^+T$ lymphocytes induced by viral infection, have been shown to explain the cholinergic-induced bronchoconstriction in asthmatic patients (Amrani and Panettieri 2002). The anticholinergics used to manage airway inflammation block the mAChRs and thereby inhibit ACh mediated reflex cholinergic bronchoconstriction (Scullion 2007).

Ipratropium bromide (Atrovent) is a short acting non-selective antagonist of M_1-M_3 receptors, which is commonly used as a therapy for COPD (Restrepo 2007). Ipratropium inhibits vagally-mediated reflexes by antagonising the action of ACh at bronchial mAChRs in the lungs which causes smooth muscle relaxation and bronchodilation, thereby facilitating airflow and alleviating exacerbations of COPD (Tranfa *et al*. 1995) and also improves pulmonary function (Restrepo

2007). Furthermore, it also inhibits the ACh-induced guanyl cyclase stimulation thereby reducing the cyclic guanosine monophosphate (cGMP) which acts as a mediator for bronchoconstriction (Pakes *et al*. 1980).

Iptratropium was introduced in 1974 and provided anticholinergic effects while avoiding the adverse side effects of atropine (Chapman 1993). It is administered by oral inhalation either via an aerosol inhaler or nebulizer. Pharmacokinetic studies have shown that following inhalation of a 2mg dose of ipratropium, only 7% of the dose is absorbed into the systemic circulation either from lungs or gastrointestinal tract (Boehringer Ingelheim 1987). Studies have also shown that oral inhalation of ipratropium bromide via a nebulizer starts to work within 15-30 minutes of administration but may take up to 90 minutes for maximal bronchodilation in COPD patients (Rebuck *et al*. 1987, Karpal *et al*. 1990). Furthermore, its duration of action is about 6 hours (Karpal 1993).

Another inhaled anticholinergic used for the treatment of COPD is tiotropium bromide monohydrate. Tiotropium is a long-acting agent and displays a higher affinity for mAChRs than ipratropium (Haddad *et al*. 1994). Although tiotropium binds to M_1-M_3 subtypes, its dissociation from the M_2 subtype is much faster thereby resulting in more selective antagonism of M_1 and M_3 mAChRs and also its prolonged pharmacologic activity provides bronchodilation for up to 24 hours (Barnes 2001). Clinical trials have revealed that COPD patients taking tiotropium once daily or ipratropium four times daily for one year improved the quality of life amongst patients but tiotropium also increased the forced expiratory volume (FEV_1) (Vincken *et al*. 2002).

Tiotropium is quickly absorbed into the systemic circulation with a peak plasma concentration within 5 minutes of administration and has a terminal half-life of 5-6 days (Barnes 2000). It is delivered by inhalation via a device specifically developed for COPD patients known as the

Spiriva® HandiHaler® (Boehringer Ingelheim Pharmaceuticals 2014). Since its approval in 2002, more than 8 million patients have used inhaled tiotropium worldwide with net sales of roughly US$2.4 billion in 2007 (Singh *et al*. 2008).

1.1.3 Side effects associated with ipratropium and tiotropium

Despite its clinical usefulness, studies have reported cardiovascular risks associated with COPD patients with underlying ischaemic heart disease (IHD) being treated with anticholinergic therapies (Singh *et al*. 2008, Ogale *et al*. 2010). Singh and colleagues found in their meta-analysis that patients assigned to randomized inhaled anticholinergics showed an increased risk for myocardial infarctions, and cardiovascular death (Singh *et al*. 2008). Similarly, observational studies have also found an increased correlation between anticholinergic use and cardiovascular events (Macie *et al.* 2008) and cardiovascular-related mortality (Lee *et al*. 2008). In addition, Ogale and colleagues identified increased risk of cardiovascular events including heart failure, acute coronary syndrome and dysrhythmia in patients exposed to anticholinergic treatment within 6 months of initiating therapy (Ogale *et al*. 2010). Furthermore, Shaik *et al*. (2012) has shown that ipratropium causes scrambling of the erythrocyte membrane and cell shrinkage which leads to apoptosis. The study reveals that ipratropium triggered cell death potentially via an increase in cytosolic calcium activity due to non-selective cation channels activation which has previously been shown to be activated by oxidative stress (Brand *et al*. 2003).

1.2 Coronary Heart Disease

Coronary heart disease (CHD) is set to be the leading cause for morbidity and mortality within the developing world by 2020 (Kloner and Rezkalla 2004). It is characterised by the narrowing or blockage of the coronary arteries, caused mostly by atherosclerosis. Atherosclerosis is a progressive condition that affects arterial blood vessels that can manifest into coronary artery

occlusion resulting in myocardial infarction. Atherosclerosis occurs as a result of the progressive development of atherosclerotic plaques that protrude into the lumen of the coronary arteries resulting in the obstruction of blood flow (Ross 1999). Plaque formation is a gradual process that can occur primarily due to a range of insults on the vascular endothelium including the accumulation of low density lipoproteins (LDL) or ‘’bad’’ cholesterol carriers. Vascular insults are implicated due to smoking, obesity, hyperlipidaemia, hypertension, diabetes and genetic predisposition (Altman 2003). These factors can lead to endothelial damage and dysfunction. Furthermore, certain white blood cells such as monocytes and T cells become activated and enter the artery’s wall transforming into foam cells. Over time, accumulation of LDL, foam cells, calcium, smooth muscle cells and cell debris leads to hardening (also known as furring) and narrowing of the arteries. This leads to the formation of atherosclerotic plaque or atheroma (Libby *et al.* 2002) which results in insufficient supply of blood to the myocardium in a process referred to as ischaemia.

Myocardial ischaemia is the inability of the myocardium to receive oxygen and nutrient supply with respect to demand (Asano *et al*. 2003). Under normal conditions the myocardium respires aerobically to produce energy in the form of ATP, which permits it to pump blood around the body. However, during myocardial ischaemia the tissues respire anaerobically to produce ATP via glycolysis (Das and Harris 1990). Anaerobic respiration also leads to the production of lactic acid and hydrogen ions (H^+) which decrease the pH leading to acidosis (Dennis *et al*. 1991). The increased levels of H^+ in the cell activates the Na^+/H^+ exchanger (NHE) for the removal of H^+ from the cell (Karmazyn and Moffat 1993). This increases intracellular Na^+ concentrations which in itself are linked to mitochondrial damage and cell swelling (Takeo and Tanonaka, 2004).

Sustained ischaemia can have detrimental effects which includes: cell swelling, oxidative phosphorylation disruption, ATP depletion, disruption of the Na^+/K^+ and Ca^{2+} pumps, intracellular pH decrease, reversible and irreversible myocyte damage and free radical mediated injury (Graham *et al*. 2004, Braunwald and Kloner 1985).

Reperfusion is the restoration of blood flow to the myocardium after an episode of ischaemia. Reperfusion is a vital process to salvage the myocardial tissue as it removes the lactic acid from the tissue and also restores pH levels. Although reperfusion is critical to salvage reversibly damaged myocytes, it has also been shown to have deleterious effects on the myocardium, thus it is termed lethal reperfusion injury (Yellon and Baxter 2000, Braunwald and Kloner 1985). Lethal reperfusion injury can lead to the development of arrhythmias, myocardial stunning, and reversible and irreversible cell damage (Bolli *et al*. 1999). Although the mechanism involved in reperfusion injury remains to be fully understood, various contributors have been proposed including oxygen derived free radicals, cell swelling, calcium overload, myocardial haemorrhage, necrosis and apoptosis.

1.2.1 Oxygen derived free radicals

The re-introduction of oxygenated blood to the ischaemic myocardium leads to the generation of injurious oxygen derived free radicals (Bolli *et al*. 1989). Free radicals are generated by the mitochondria and exist within cells. They are catabolised by scavengers such as superoxide dismutase and glutathionine peroxidase (Forde and Fitzgerald 1997). In the non-ischaemic myocardium these free radical scavengers limit free radical dependent injury via their antioxidant abilities whereas during ischaemia reperfusion the levels of free radicals such as superoxide anion (O_2^-) and hydroxyl radical reach peak levels (Zweier *et al*. 1987). In addition, during

respiratory burst activated neutrophils can utilise nicotinamide adenine dinucleotide phosphate (NADPH) to reduce oxygen producing oxygen free radicals such as O_2- (Segal 2005).

Studies have shown that reoxygenation leads to a profound increase in the levels of oxygen derived free radicals which mediate functional injury to the myocardium and also impaired contractile function in isolated heart (Zweier 1988, Bolli *et al*. 1989). Free radicals have been shown to generate lipid peroxides, which can lead to the disruption of membrane integrity, inhibition of membrane enzymes and structural changes (Forde *et al*. 1999). The use of free radical scavengers has been shown to decrease free radical dependent injury of the myocardium in different models (Jolly *et al*. 1984, Stewart *et al*. 1983). The administration of antioxidants such as superoxide dismutase and catalase, have shown to minimise free radical injury in the ischaemia reperfusion model of myocardium in dog (Jolly *et al*. 1984). Bognar *et al*. 2006 showed that a modified superoxide dismutase, HO-3538, decreased the release of reactive oxygen species and also limited the opening of the mitochondrial permeability transition pore thereby preventing the release of pro-apoptotic proteins such as cytochrome c.

1.2.2 Calcium overload

Myocardial contractility is primarily controlled by calcium cycling into and out of the cytoplasm of cardiac myocytes as well as calcium sensitivity of various proteins in cardiac myocytes. Calcium enters the cardiac myocyte cytoplasm from the extracellular space mainly through L-type calcium channels which triggers calcium ions (Ca^{2+}) release from the ryanodine receptors (RyR) in the sarcoplasmic reticulum, in a process called calcium-induced calcium release (CICR) (Treinys and Jurevicius 2008). Ca^{2+} binds to troponin C which is part of a troponin complex that together with tropomyosin affects the interaction between actin and myosin leading

to the development of myocardial contraction (Kobayashi *et al*. 2008). The interaction between troponin C and Ca^{2+} is a critical step of calcium induced control of myocardial contractility. Under normal conditions, Ca^{2+} is removed from the cytoplasm to the outside of the cell by a sodium–calcium (Na^{+}/Ca^{2+}) exchanger (Sher *et al*. 2008). The Na^{+}/Ca^{2+} exchanger uses energy that is stored in the electrochemical gradient of sodium by allowing Na^{+} to flow down its concentration gradient across the plasma membrane into the cell in exchange for the Ca^{2+}. The Na^{+}/Ca^{2+} exchanger imports 3 Na^{+} in to the cell and exports one Ca^{2+} outside the cell and can efficiently transport up to 5000 Ca^{2+} per second (Carafoli *et al*. 2001). The Na^{+}/Ca^{2+} exchanger requires an active Na^{+}/K^{+} ATPase which keeps the internal sodium levels low. A plasma membrane calcium ATPase can also aid in the removal of cytoplasmic Ca^{2+} to the outside (Oceandy *et al*. 2007). The regulation of Ca^{2+} in a normal cardiac myocyte is shown in figure 1.1. Reperfusion to the ischaemic myocardium has been shown to cause an accumulation of intracellular Ca^{2+} leading to calcium overload (Duchen 2000, Valverde e*t al.* 2008). Ischaemia reperfusion injury reduces the activity of the sarcolemmal Na^{+}/K^{+} ATPase and activates the Na^{+}/Ca^{2+} exchanger activity in a reverse mode allowing Ca^{2+} entry into the cell (Dhalla *et al*. 2007). Furthermore, ischaemia reperfusion also adversely effects the Ca^{2+} proteins in the sarcoplasmic reticulum and sarcolemma including sarcoplasmic reticulum Ca^{2+}-ATPase and L-type calcium channels thereby contributing to the an intracellular Ca^{2+} overload (Dhalla *et al*. 2007).

Calcium overload is also believed to activate enzymes such as proteases, lipases and nucleases which can induce electrical and structural damage to the cell membrane and sarcolemmal membrane (Atsma *et al*. 1995, Yoshida *et al*. 1993). Calcium overload also leads to the opening

of the ATP-sensitive potassium (K_{ATP}) channel and mitochondrial permeability transition pore (MPTP) therefore activating apoptosis (Makazan *et al*. 2007, Minezaki *et al*. 1994).

Ischaemia reperfusion mediated calcium overload can also lead to the hypercontracture of cardiac myocytes (Rodríguez-Sinovas *et al*. 2007). This can cause abrupt shortening of cell length accompanied by cytoarchitectural disorganisation leading to cytoskeletal damage. Hypercontracture of cardiac myocyte can result in cell necrosis and is referred to as contraction band necrosis (Rodríguez-Sinovas *et al*. 2007).

Figure 1.1: Calcium entry and exit from a normal cardiac myocyte (Marin-Garcia 2010).

■ = Calcium, $I_{ca,L}$ = L-type calcium channel, SERCA = Sarco/Endoplasmic reticulum Ca^{2+} ATPase.

1.2.3 Apoptosis

Apoptosis is a natural energy dependent cell death process that prevents an inflammatory response by disposing of cells that are not required in an organism (Czerski and Nunez 2004). It is important to mention that apoptosis differs from necrosis which is the death of living cells and tissue. The latter is generally detrimental to an organism whereas apoptosis benefits an organism in many ways. Apoptosis can be stimulated in response to different factors, which may either be regulated by extrinsic or intrinsic signals. Extrinsic signals move across the plasma membrane or go through signal transduction to result in apoptosis. The extrinsic signals include toxins, hormones, growth factors, nitric oxide and cytokines (Borutaite *et al*. 2003). In contrast, the intrinsic signals include cellular stress that initiate intracellular apoptotic signaling that commits a cell to perform "suicide".

There have been numerous studies that relate apoptosis with ischaemia reperfusion injury. It is not entirely clear whether apoptosis is initiated in ischaemia, reperfusion or both. Chakrabarti *et al* (1997) proposed that apoptosis was activated in isolated rat hearts during brief ischaemia without reperfusion. However, some research has also demonstrated that apoptosis is activated during ischaemia and accelerates during reperfusion (Gottlieb and Engler 1999). Although there is a controversy regarding when exactly apoptosis occurs, the past research provides strong evidence to relate apoptosis with the ischaemia reperfusion injury.

There are two main apoptotic pathways called the extrinsic and the intrinsic pathways. The extrinsic pathway is also called the death receptor pathway. It involves an apoptotic stimulus to activate the extracellular death receptor such as Fas and Tumour necrosis factor receptor-1 (TNFR1) (Borutaite *et al*. 2003). A protein known as Fas ligand (FasL) binds to its receptor (Fas)

and results in oligomerisation (Caulfield and Latham 2014). Apoptosis is initiated as the death receptor is activated, which causes an association of the receptor with Fas associated death domain (FADD) protein. FADD then binds to pro-caspase 8 through the proteins death effector domain (DED). The complex then activates caspase 8 which binds to caspase 3 initiating apoptosis (Kelley *et al.* 2010, Caulfield and Latham 2014). Caspase 3 leads to various biochemical and morphological changes characteristic of apoptosis by cleaving various death substrates. Death inducing signalling complex (DISC) is the term given to the complex of Fas, FADD and caspase 8. The process of the extrinsic apoptotic pathway is summarised in Figure 1.2.

Figure 1.2: Extrinsic apoptotic pathway (Rieux-Laucat *et al.* 2003). DISC = Death inducing signalling complex (DISC), FADD = Fas associated death domain, FasL = Fas ligand

The other apoptotic pathway is the intrinsic or mitochondrial pathway that occurs in response to various intracellular stress signals (Lopez *et al*. 2006). These include DNA damage, hypoxia, nutrient deprivation, oncogenes, and survival factor deprivation. The Bcl-2 family of proteins play a key role in this pathway to regulate the mitochondrial membrane permeability (Lopez *et al*. 2006). Protein 53 (p53) plays a critical role in the activation of the intrinsic pathway. It is a tumour suppressor and has been termed as "guardian of the genome" as it conserves stability and prevents gene mutations (Ashkenazi 2002). Cellular stress such as DNA damage is detected by p53 and if the damage is non-repairable, it commits to apoptosis.

The Bcl-2 proteins are essential in the intrinsic pathway and they consist of both anti-apoptotic and pro-apoptotic members. The pro-apoptotic members include multi-domain BH 1-3 proteins such as Bax, Bak and Bok and the BH-3 only proteins such as Bid, Bad and p53 upregulated modulator of apoptosis (PUMA). Whereas, the anti-apoptotic members include Bcl-2, Bcl-XL and Mc1-1which exhibit BH1-4 homology domains (Kim *et al*. 2006). Upon the reception of a stress signal, the pro-apoptotic proteins are activated and the anti-apoptotic proteins are inactivated. The expression of pro-apoptotic proteins leads to the destabilisation of the mitochondrial membrane and initiates cytochrome c release which binds to adaptor protease activating factor-1 (Apaf-1) forming apoptosome (Chang *et al*. 2003). Apoptosome functions to regulate the catalytic activity of caspase 9, which in turn activates the main downstream effector caspase, such as caspase 3, that play a vital role in regulation of cellular apoptosis. Other intra-mitochondrial proteins such as second mitochondria-derived activator of caspases (SMAC) and apoptosis inducing factor (AIF), which act on caspases (caspase 3) and execute apoptotic cell death machinery are also released (Chang *et al*. 2003). Namura *et al*. (1998) showed increased levels of activated caspase 3 with peak at between 30-60 minutes of reperfusion in a cerebral model of ischaemia reperfusion injury. Opposingly, anti-apoptotic protein overexpression results in inhibition of cytochrome c release and therefore supports survival (Majno and Joris 1995). The process of intrinsic apoptotic pathway is summarised in figure 1.3.

Figure 1.3: Chemotherapy/radiotherapy-induced intrinsic apoptotic pathway (Ashkenazi 2002). Apaf-1 = adaptor protease activating factor-1, SMAC = Second mitochondria-derived activator of caspases, PUMA = p53 upregulated modulator of apoptosis, Bcl-2 = Anti-apoptotic members of the Bcl-2 family of proteins, Bak = Pro-apoptotic members of the Bcl-2 family of proteins.

1.2.4 Necrosis

Necrosis is an energy independent cell death process during which the cells swell, and the plasma membrane collapses followed by the lysis of cellular organelles (Proskuryakov *et al*. 2002). Necrosis can be caused by various factors including infections, toxins and trauma that lead to an unregulated cell component digestion (Proskuryakov *et al*. 2003). The cell membrane integrity of the necrotic cells is lost and there is an uncontrolled release of cell death products into the intracellular space which triggers an inflammatory response in the surrounding tissue (Kasper and Zaleznik 2001). This prevents the leukocytes i.e. phagocytes, from carrying out phagocytosis and eliminating the dead cells. It is therefore important to remove necrotic tissue in clinical conditions by a process known as debridement as untreated necrosis leads to a build-up of decompsoing dead tissue and cell debris at the site of cell death such as in gangrene (Kasper and Zaleznik 2001). The structural cellular differences between apoptosis and necrosis are summarised in figure 1.4.

Necrosis is the predominant form of cell death in ischaemia which leads to oxygen and glucose depletion. It results in death of endothelial and non-proliferating cells (Proskuryakov *et al*. 2002). Wang *et al*. (2008) showed that IR causes cell death via necrosis of isolated cells from rat skeletal muscle. Necrosis does not only take place in pathological conditions but also during some normal physiological processes. For example, necrosis is involved in the follicular maturation in the process of oogenesis and also during small intestine renewal (Mayhew *et al*. 1999, Murdoch *et al*. 1999).

Figure 1.4: Cellular structural differences between the progression of the processes of apoptosis and necrosis. Necrosis results in irreversible cell injury characterised by dense clumping, disruption to cell organelles and membrane breakdown (Adapted from Hendricks 2002).

1.2.5 Autophagy

Autophagy is a catabolic process that results in the degradation of intracellular organelles and cytoplasmic contents (Yang and Klionsky 2010). Nutrient deprivation usually activates the process but it has also been linked with physiological and pathological processes such as cellular differentiation, cell development, stress and cancer (Levine and Kroemer 2008). As opposed to apoptosis and necrosis, it is an energy producing process and provides an alternative source of energy under stress conditions such as DNA damage, nutrient deprivation and build-up of ROS (Klionsky 2007). The key regulator of autophagy is the mammalian target of rapamycin (mTOR)

kinase. During nutrient deficiency it functions as a pro-survival mechanism but uncontrolled or excessive autophagy can lead to cell death.

1.3 Myocardial injury signaling pathways

Mitogen activated protein kinases (MAPK) have been implicated as important signaling cascades involved in cardiac cell death in response to ischaemia-reperfusion (IR) injury (Abe *et al*. 2000, Yue *et al*. 2000, Lips *et al*. 2004). Activation of extracellular signal regulated kinase (ERK 1/2) has been shown to provide myocardial protection against IR injury in mice (Das *et al*. 2009), rat cardiac myocytes (Wang *et al*. 2012), and in rabbit hearts (Yang *et al*. 2011). ERK 1/2 have also been identified as central components of the reperfusion injury salvage kinase (RISK) pathway which has been shown to protect myocardium from IR injury (Hausenloy and Yellon 2004). Interestingly, the inhibition of p38 MAPK has been shown to reduce myocardial IR injury in mice (Gao *et al*. 2001) and inhibition of c-Jun NH (2)-terminal protein kinase (JNK) has been shown to protect myocardium against IR injury in rats (Chambers *et al*. 2012).

Furthermore, the phosphatidylinositol-3-kinase (PI3K) has also been shown to be involved as an important cellular mediator in response to IR injury. Signalling via PI3K and subsequent activation of Akt has been shown to protect against IR injury (Fujio *et al*. 2000). Akt has also been identified as a central component of the RISK pathway, which provides protection to the myocardium against the IR injury (Hausenloy and Yellon 2004). The activation of Akt has been demonstrated to activate mitochondrial Raf-1 (Majewski *et al*. 1999) which phosphorylates and inactivates the pro-apoptotic factor, BAD (Wang *et al*. 1996).

1.3.1 Mitogen Activated Protein Kinases

MAPK mediate various cellular processes including cellular growth, movement, proliferation and apoptosis (Raman *et al*. 2007, Lowes *et al*. 2002). MAPK are serine and threonine kinases

which can be activated by G protein-coupled receptors (GPRCs) and also by tyrosine kinases (Pearson *et al*. 2001). A signal such as a growth factor, cytokine or neurotransmitter activates MAPK via binding to its protein tyrosine kinase receptor. Epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) are the best-known receptors of the MAPK but G-proteins are also involved in the activation process (Cantrell 2003).

Binding of a ligand to EGF receptor induces receptor oligomerization which results in juxtaposition of the cytoplasmic and catalytic domains which allows activation of the kinase activity and transphosphorylation (Schlessinger 2000). Adaptor proteins such as Grb2 recognise sequence homology (SH2) domains such as Shc, which recruits guanine nucleotide exchange factors (GEFs) like SOS-1 to the cell membrane (Schlessinger 2000). The GEF interacts with Ras protein to induce a conformational change and the exchange of GDP for GTP, activating Ras. Raf is then recruited via binding to the switch I domain of Ras and also by lipid binding to the cell membrane (Marais et al. 1995).

Raf is a member of a family of serine/threonine kinases that includes Raf-1, A-Raf and B-Raf. Raf is the best-characterised Ras effector and its activation stimulates a signalling cascade by phosphorylation of MAPK (Liebmann 2001). MAPK in turn phosphorylates and activates downstream proteins including ERK1 and ERK2. Phosphorylation of ERK1/2 at Thr_{202} and Tyr_{204} residues transforms ERK1/2 to its active form (Butch and Guan 1996). ERK1 and ERK2 phosphorylate and activate various nuclear transcription factors and kinases including Elk-1, c-Ets1, c-Ets2, p90RSK1, MNK1, MNK2, and other proteins. Many of the targets of MAPK/ERK have been implicated in Ras induced cell transformation (Liebmann 2001).

Other member of the MAPK superfamily include JNK/SAPK (c-Jun NH (2)-terminal protein Kinase/stress-activated protein kinase) which is actively involved in cell differentiation, growth,

cell survival and death. JNK/SAPK is activated in response to environmental stresses including heat shock, UV radiation, osmotic shock, inflammatory cytokines, growth factors and GPCR agonists (Nishina *et al*. 2004). A variety of environmental stimuli impact the small GTPases of the Rho family (Rho, Rac, Cdc42), that leads to the activation of membrane protein components such as MEKKs, ASK1, TAK1/AB1 or MLK3. These protein kinases then phosphorylate and activate MKK4/7, which mediates the activation of the JNK/SAPK family members (Wada *et al*. 2004). There are three isoforms of JNK/SAPK family which include: JNK1, JNK2 and JNK3. Upon activation, JNK/SAPK may translocate to the nucleus and regulate the activity of various transcription factors such as c-Jun, ATF-2, SMAD4, p53 and Elk1 (Wada *et al*. 2004).

Furthermore, mAChRs have been shown to signal via MAPK in various studies. The M_1 receptor has been reported to activate MAPK via a pathway involving pertussis toxin-insensitive G_q or pertussis toxin-sensitive G_o G-proteins, protein kinase C (PKC) and Raf but independent of Ras (Hawes *et al*. 1995, Van Biesen *et al*. 1996). Some studies have also implicated βγ-subunits in Ras-dependent activation of MAPKs by M_1 receptor (Crespo *et al*. 1994). The M_2 subtype has been shown to activate MAPKs via release of βγ-subunits from pertussis toxin-sensitive G-proteins with subsequent tyrosine phosphorylation of Shc, leading to the activation of Ras (Winitz *et al*. 1993, Koch *et al*. 1994).

1.3.2 Phosphatidylinositol-3-Kinase

The phosphatidylinositol-3-kinase (PI3K) is a serine/threonine kinase that is involved in the regulation of cell proliferation, growth and survival (Engelman *et al*. 2006). Receptor tyrosine kinases such as cytokines, growth factors and GPCRs bind to their receptors and activate PI3K. The activated PI3K induces the conversion of phosphatidylinositol (3,4)-bisphosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) which subsequently lead to the

phosphorylation and activation of Akt (Protein kinase B, PKB) and its upstream activator PDK1 (Alessi *et al*. 1997).

Akt and PIP_3 bind at the plasma membrane and lead to the phosphorylation of Thr_{308} by PDK1 that partially activates Akt. This phosphorylates and inactivates proline-rich Akt substrate of 40 (PRAS40) and tuberous sclerosis protein 2 (TSC2) and subsequently activates mTORC1 which phosphorylates Akt at Ser_{473} to fully activate it (Sarbassov *et al*. 2005, Vander Haar *et al*. 2007). Fully activated Akt can lead to the phosphorylation of substrates in the cytoplasm and the nucleus. It can modulate cell survival via recruitment of numerous downstream effector proteins phosphorylating forkhead transcription factors, eNOS (endothelial nitric oxide synthase), and the pro-apoptotic protein of the Bcl 2 (B cell lymphoma/leukaemia 2) family BAD (Bcl-2/Bcl-XL-associated death promoter). BAD in its phosphorylated form binds to 14-3-3- proteins therefore BAD's ability to associate to Bcl-2 and Bcl-xl is diminished preventing BAD dependent apoptosis initiation (Hausenloy and Yellon. 2004). Akt also inhibits the pro-apoptotic forkhead box O (FoxO) transcription factors and contributes to cell survival (Zhang *et al*. 2011). Furthermore, Akt can activate numerous effector proteins like glycogen synthase kinase 3β (GSK3β) and stimulate glycogen synthesis as well as proteins involved in cell proliferation and growth (Hausenloy and Yellon 2004; Park *et al*. 2006). Akt in its active state is involved in mediation of other cellular fucntions such as angiogenesis, metabolism, growth and proliferation (Engelman *et al*. 2006).

1.3.3 G protein coupled receptors

G protein coupled receptors (GPCRs) also known as seven-transmembrane domain receptors represent the largest family of membrane proteins in the human genome that are involved in the activation of signal transduction pathways and cellular responses (Kobilka 2007). GPCRs consist of seven hydrophobic transmembrane segments, with an extracellular amino terminus and an intracellular carboxyl terminus (Kobilka 2007). GPCRs are activated by a spectrum of structurally diverse ligands including hormones, neurotransmitters and odour molecules (Trzaskowski *et al*. 2012).

GPCRs associate with heterotrimeric guanine nucleotide-binding proteins (G-proteins). G-protein is a trimer consisting of α, β and a γ sbunit that are tethered at the surface of the membrane by covalently attached lipid molecules. When a ligand binds to the GPCR, it causes a conformational change in the GPCR and activates the G-protein thereby exchanging its bound GDP for a GTP (Wettschureck and Offermanns 2005). The G_{α} subunit with the attached GTP becomes activated and dissociates from the $G_{\beta\alpha}$ complex to affect intracellular signaling proteins such as enzymes that act as second messengers to regulate a cellular response. The $G_{\beta\alpha}$ complex also diffuses along the inner membrane surface to activate proteins such as ion channels.

The different classes of G-proteins consist of different types of α subnuits which regulate specific signal transduction pathways. The main signal transduction pathways involving G-proteins include the cyclic adenosine monophosphate (cAMP) signal pathway and the phosphatidylinositol signal pathway (Wettschureck and Offermanns 2005).

The G_{α} subunit has intrinsic GTPase activity that hydrolysis GTP to cause re-association of inactive G_{α} subunit bound to GDP to the $G_{\beta\alpha}$ complex which can again bind to a GPCR and await activation. The process of G-protein mediated signal transduction is shown in figure 1.5.

Figure 1.5: G-protein mediated signal transduction (Rasmussen *et al*. 2011).

1.4 Muscarinic Receptors

Muscarinic acetylcholine receptors (mAChRs) mediate various actions of ACh in the central nervous system (CNS) and peripheral nervous system (Van Koppen and Kaiser 2003). mAChRs belong to metabotropic family of receptors that use G- proteins to initiate signal transduction. mAChRs regulate secondary messenger pathways via an ion channel and enzyme activation coupled to the G-proteins. In mammals, five distinct mAChR subtypes (M_1-M_5) have been recognised (Bonner 1989, Hulme *et al*. 1990, Caulfiled and Birdsall 1998) with each subtype being the product of a different gene. The M_1, M_3 and M_5 receptor subtypes preferentially couple to the pertussis toxin-insensitive $G_{\alpha q/11}$, $G_{\alpha 13,}$ $G_{\alpha 14}$, and $G_{\alpha 16}$ subtypes of G protein that leads to the activation of phospholipase C (PLC), phospholipase A2 (PLA2), phospholipase D (PLD), tyrosine kinase and calcium influx while M_2 and M_4 subtypes preferentially couple to G_i and G_o

proteins which lead to the inhibition of adenylyl cyclase (AC) (Caulfield1993). Table 1 summarises the distribution, function and mechanism of action of mAChRs.

Table 1: Summary of muscarinic receptor distribution, function and mechanism of action (Adapted from Sellin *et al*. 2008).

1.4.1 M_1 receptors

The M_1 mAChRs are members of the G-proteins coupled receptor (GPCR) family and mediate metabotropic functions of ACh in the central nervous system. They are mainly present in the brain with high expression levels in the cerebral cortex and hippocampus and are involved in various important processes such as cognition and memory (McKinney and Coyle 1991, Ladner and Lee 1998). In the airways, these receptors are localised to parasympathetic ganglia and are involved in slow ganglionic transmission (Celli 2004). The M_1 receptors thereby enhance cholinergic reflex bronchoconstriction. The M_1 subtype mAChR is bound to the pertussis toxin-insensitive G_q proteins and signal via activation of phospholipase C (PLC) (Caulfield 1993).

They are involved in various cellular processes including control of seizures and maintaining cognitive processes such as memory and learning (Marino *et al*. 1998). Furthermore, activation of M_1 receptor has been shown to decrease harmful β-amyloid peptide secretion thereby providing a therapeutic target for Alzheimer's disease (Eglen 2005). It is encoded by human gene CHRM1 and is localised within chromosome at 11q13 (Bonner *et al*. 1991).

1.4.2 M_2 receptors

The M_2 receptors belong to the GPCR family and are bound to the G_i proteins and their activation leads to the inhibition of adenylyl cyclase (AC) (Caulfield1993). They are predominantly present in the heart where they are involved in decreasing cardiac beat and reduced atrial contractility (Wess *et al*. 1990, Brown and Taylor 1996). The βα subunits of the G protein coupled to the M_2 receptor opens K^+ channels in the heart causing an outward K^+ current thereby reducing the heart rate (Boron and Boulpaep 2005). The M_2 receptors are present on the cholinergic nerve endings and their antagonism results in an increased release of ACh and thereby bronchoconstriction to cholinergic nerve stimulation (Celli 2004). It is encoded by human gene CHRM2 and is localised within chromosome at 7q35-q36 (Bonner *et al*. 1991).

1.4.3 M_3 receptors

The M_3 mAChRs are bound to the G_q proteins and mediate cellular processes via activation of phosphoinositide phospholipase C (PLC) (Caulfield 1993). These receptors are predominantly present in the smooth muscles, lungs, endocrine glands, exocrine glands and brain (Lin *et al*. 1997, Weiner *et al*. 1990). The activation of the M_3 receptors is mainly involved in smooth muscle contraction of the airways which leads to bronchoconstriction (Eglen *et al*. 1996). The

M_3 receptor is thereby an important therapeutic target to treat pulmonary obstructions such as COPD. Furthermore, these receptors are also present on β cells of the pancreas and may also be involved in regulation of glucose homeostasis and insulin release (Gautam *et al*. 2006, Weston-Green *et al*. 2006). The M_3 is encoded by the human gene CHRM3 and its chromosomal location is 1q43 (Bonner *et al*. 1991).

1.4.4 Adenylyl Cyclase

Adenylyl cyclase (AC) is an enzyme located in the plasma membrane of cells that plays an important role in the regulation of various cellular signaling pathways. AC catalyses the transformation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) which is released into the cytoplasm and acts as a second messenger (Zhang *et al*. 1997). cAMP relays extracellular signals to intracellular effectors and initiates a signaling cascade which can activate other protein kinases thereby opening ion channels or exposing the active sites of other regulatory proteins (Zhang et al. 1997).

The even numbered mAChRs have been shown to couple with and inhibit AC activity (Baumgold 1992). Furthermore, Parker *et al*. 1991 showed that the $G_{\alpha i}$ subtype attached to mAChRs is responsible for this response. The regulation of AC activity by mAChRs can be regulated via calcium and protein kinase mechanisms (Jansonn *et al*. 1991, Baumgold 1992). Protein Kinase A (PKA) is one of the major targets of cAMP (Gerits *et al*. 2008). It is a serine/threonine protein kinase that consists of two regulatory and two catalytic subunits in its inactive form. Each regulatory unit consist of two binding sites for cAMP and upon its binding, the PKA holoenzyme dissociates releasing the two catalytic subunits which possess the protein kinase activity (Skålhegg and Tasken 2000). Apart from PKA, phosphodiesterases (PDE) are also a target for cAMP and they catalyse the conversion of cAMP to AMP (Houslay 2006)

thereby comprising a feedback mechanism that can turn off the cAMP/PKA pathway (Gerits *et al*. 2008). Figure 1.6 summarises the mechanism of AC signal transduction.

mAChR can also couple to AC via a calcium regulation mechanism in which cAMP could be produced via calcium/calmodulin sensitive (type I and type III) or insensitive (type II, IV, V and VI) adenylyl cyclases. The coupling of mAChRs to AC depends on the type of cell line they are expressed in. However, studies in various cell types have shown that cAMP accumulation occurs via mAChR mediated phosphodiesterase inhibition (Meeker and Harden 1982). Interestingly, the odd numbered mAChRs i.e. M_1 and M_5 have also been shown to weakly stimulate AC activity in a few studies (Stein *et al*. 1988, Ashkenazi *et al*. 1989).

Figure 1.6: Adenylyl cyclase signal transduction mechanism (adapted from Moran et al. 2012). GDP = Guanosine diphosphate, GTP = Guanosine triphosphate, ATP = Adenosine triphosphate, cAMP = Cyclic Adenosine 3',5'-monophosphate, AMP = Adenosine monophosphate.

1.4.5 Phospholipase C

PLC is a class of enzyme that plays an important role in transmembrane signaling. PLC results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) as secondary messengers upon an extracellular stimulus such as a growth factor, hormone or a neurotransmitter (Singer *et al*. 1997). The secondary messenger IP_3 is released into the cytosol where it binds to IP_3 receptors, particularly the calcium channels in smooth endoplasmic reticulum. This consequently increases the calcium levels in the cytosol which regulates an intracellular cascade (Alberts *et al*. 2002). The other secondary messenger DAG remains bound to the membrane and is involved in the activation of protein kinase C (PKC) which in turn phosphorylates different enzymes resulting in altered cellular activity (Alberts *et al*. 2002). Figure 1.7 demonstrates the role of PLC in calcium release from cytoplasmic storage.

There are various forms of PLCs which have been recognized and different subunits of G-protein activate them. These include PLCβ, PLCγ, PLCδ and PLCε (Smrcka *et al*. 1991, Taylor *et al*. 1991). The activation of PLC by ACh via odd numbered mAChRs has been well documented (Lambert *et al*. 1992). In the M_1 subtype PIP2 is broken down by the PLCβ1 via Gq/11 α subunits whereas PLCβ and PLCγ are involved in the M_5 subtype (Berstein *et al*. 1992, Sawaki *et al*. 1993). The activation of PLCγ has been shown to be by phosphorylation of the tyrosine kinase via the M_5 mAChRs. The M_5 mAChRs mediates influx of calcium activating voltage-independent calcium channels, consequently phosphorylating the tyrosine kinase (Gusovsky *et al*. 1993).

However, studies have also shown that the even numbered mAChRs can weakly stimulate PLC

via Gα2 and Gαi3, PTX-sensitive G-proteins (Ashkenazi *et al*. 1989, Dell'Acqua *et al*. 1993).

Figure 1.7: The Role of Phospholipase C in release of Calcium from cytoplasmic storage (Marr and Acorn 2007). PIP_2 = phosphatidylinositol 4, 5-bisphosphate, IP_3 = inositol 1,4,5-triphosphate, DAG = diacylglycerol.

1.5 Acetylcholine

ACh is the primary neurotransmitter of the parasympathetic nervous system (PNS) and is formed by the enzyme choline acetyl transferase (ChAT) in the nerve endings (Gosens *et al*. 2006). Choline is co-transported with sodium into the cytoplasm of the cholinergic neuron via an energy-dependent carrier system from the extra-cellular fluid (Siegel *et al*. 1999). The uptake of choline is a rate-limiting step where the enzyme choline acetyltransferase catalyses the formation

of ACh in the cytosol from choline and acetyl coenzyme A (CoA) (Parsons *et al*. 1987). The acetyl CoA is derived from the mitochondria and is produced by the Krebs cycle (Siegel *et al*. 1999).

The ACh is then packaged into storage vesicles via active transport coupled to the efflux of protons (Parsons *et al*. 1987). Upon membrane depolarisation the pre-synaptic voltage-sensitive calcium channels open and cause an increase in the levels of intracellular calcium which promotes the fusion of synaptic vesicles with the cell membrane (Varoqui *et al*. 1996). This causes the release of ACh from the synaptic vesicles which could bind to postsynaptic receptors such as muscarinic and nicotinic receptors or the cholinergic nerve terminals (Racke and Matthiesen 2004). Once released, the functional effects of ACh are terminated by its degradation by the enzyme acetylcholinesterase (AChE) in the synaptic cleft to non-active choline (Gwilt *et al*. 2007).

ACh and ChAT have been shown to be present in various non-neuronal and immune system cells including endothelial cells, smooth muscle cells, lymphocytes and macrophages (Wessler *et al*. 1999). ACh has been linked to the regulation of basic cellular processes including proliferation, cell differentiation, cytoskeleton organization and transport of ions and water via paracrine and autocrine mechanisms (Wessler *et al*. 2003). Unlike neuronal cells, the non-neuronal ACh is released via active transport mediated by organic cation transporters and its inhibition or reduced levels have been linked with pathogenesis of certain diseases such as cystic fibrosis (Wessler *et al*. 2007).

ACh is the most important neurotransmitter of the parasympathetic nervous system and acts to stimulate the contraction of the smooth muscle in the airways and is also involved in mucus

secretion (Gosens *et al*. 2006). In addition, ACh is suggested to play a key role in airway remodeling regulation during chronic airway inflammation (Gosens *et al*. 2005).

1.5.1 Clinical relevance of mAChRs

The mAChR agonists or antagonists have been approved for use of various pathophysiological conditions including gastrointestinal disorders, urinary bladder disorders and respiratory diseases (Wess *et al*. 2007). However, their clinical usefulness has been limited due to the side effects associated with these agents which result in non-selective activation or blockade of certain or all mAChRs. The orthosteric binding sites for ligands have a highly conserved amino acid sequence within the five subtypes, and therefore this has become a hindrance in the development of subtype-selective agents (Hulme *et al*. 2003). However, the potential clinical usefulness of mAChRs is vast and their involvement in some conditions will be summarised below.

1.5.2 Alzheimer's disease and cognitive impairments

Studies have shown that mAChRs play significant roles in facilitating cognitive functions (Hasselmo 2006). Acetylcholinesterase inhibitors that increase brain ACh levels are important pharmacotherapy agents for Alzheimer's disease (Lleo *et al*. 2006). There is pharmacological evidence that has demonstrated the cognition-enhancing effects of ACh mediated by the M_1 mAChR (Messer 2002, Fisher *et al*. 2003). Marino *et al*. (1998) showed in hippocampal pyramidal cells that the M_1 mAChR potentiates N-Methyl-D-aspartate (NMDA) receptor currents. As NMDA receptor activity is crucial in mechanisms such as learning and memory, M_1 mAChR may exert their cognition-enhancing effects via potentiating NMDA receptor currents (Marino *et al*. 1998). In addition, the M_1 mAChR subtype is the largest population in higher brain areas, which are critically involved in cognitive processes (Levey 1993).

Studies have also shown that the activation of M_1 receptor signalling pathway may inhibit the deposition of β-amyloid peptide, which is an important pathological feature of Alzheimer's disease (Eglen 2005). There has been a great effort in developing selective M_1 agonists to manage Alzheimer's disease. Despite the cognition enhancing effects observed with these agents, the majority of clinical trials involving compounds such as alvameline, milameline, xanomeline, have either been discontinued or are on hold. This is either because of the adverse side effects associated with these compounds or due to the lack of efficacy in patients with dementia (Eglen 2005). The major obstacle in progression in this field has been that these compounds do not exhibit M_1 receptor selectivity *in vivo*. This leads to non-selective mAChR mediated side effects thereby limiting the drug dose and subsequently preventing therapeutic effect.

1.5.3 Overactive bladder

Overactive bladder (OAB) is a chronic and debilitating disease that gives rise to urinary symptoms of frequency, urgency and urges incontinence, which contributes to a significant impairment to a patient's quality of life (Chapple 2000). The symptoms of OAB are linked to involuntary contractions of the detrusor muscle during bladder filling. mAChR antagonists are used as the pharmacological therapy for the treatment of OAB. ACh induced stimulation of post-ganglionic mAChRs is involved in both normal and involuntary bladder contraction (Andersson 1993). The smooth muscles in the human urinary bladder consist of the M_2 and M_3 subtypes of which the M_2 has the predominant population (Wang *et al*. 1995). However, both the subtypes have been shown to mediate bladder contraction (Hegde and Eglen 1999).

Activation of M_3 subtype by ACh leads to hydrolysis of phosphoinositol, accumulation of intracellular calcium and subsequently contraction of smooth muscle (Chapple 2000). Whereas

the activation of M_2 results in the inhibition of adenylate cyclase which causes smooth muscle contraction by indirect inhibition of sympathetically (β-adrenoreceptor)-mediated augmentation of cAMP levels and bladder relaxation (Hegde *et al*. 1997). Therefore, during micturition, the M_3 subtype activation induces direct smooth muscle contraction whereas the activation of M_2 subtype reverses sympathetically mediated smooth muscle relaxation, the end result being more efficient voiding of urine (Hegde and Eglen 1999).

One of the pharmacologic agents used in the treatment of OAB is Oxybutynin, which is a potent mAChR antagonist selective for the M_1 and the M_3 receptor subtypes (Nilvebrant and Sparf 1986). Oxybutynin has been shown to competitively inhibit carbachol and ACh induced contractions of isolated urinary bladder from various species (Waldeck *et al*. 1997). It has also been shown to evoke contraction of guinea pig and human detrusor muscle (Yono *et al*. 1999, Tonini *et al*. 1987). Although Oxybutynin has been shown to be clinically effective, it causes adverse side effects including dry mouth, constipation, drowsiness and blurred vision (Chapple 2000). This often leads to poor patient compliance, treatment discontinuation or dose reduction to a level of minimal clinical benefit.

However, there has been more success using Tolterodine, which was developed using a functional approach to achieve bladder selectivity rather than mAChR subtype selectivity (Chapple 2000). Tolterodine is a potent competitive muscarinic receptor antagonist (M_1-M_5) that has been shown to have equivalent therapeutic efficacy to oxybutynin, but improved tolerability (Nilvebrant *et al*. 2000). Its equivalent therapeutic efficacy at the recommended dosage allows long-term treatment, as there is lower incidence and severity of dry mouth, less need for dosage reduction and fewer treatment withdrawals (Chapple 2000).

1.5.4 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a gastrointestinal disorder characterised by unexplained abdominal pain, discomfort and bloating in association with altered bowel habits (Andresen and Camilleri 2006). The pathophysiology of IBS depends on a number of factors including abnormal gastrointestinal motor function, visceral hypersensitivity, psychological factors, autonomic dysfunction and mucosal inflammation (Andresen and Camilleri 2006, Houghton *et al*. 2005). There is a considerable burden of illness associated with IBS as studies have shown that IBS patients have reported lower quality of life, missed more days off work and consume more health service resources (Akehurst 1999).

Traditional therapy for IBS ranges from modifications in diet to pharmacological intervention and is often unsatisfactory. The most common drugs used are the antispasmodics of which the muscarinic antagonist agents are an important example (Houghton *et al*. 1997). Zamifenacin, a selective M_3 subtype antagonist has been shown to reduce distal colonic motility in patients with IBS. It also did not provide atropine-like side effects that are usually associated with such drugs, thereby showing greater gut selectivity than conventional anticholinergics (Houghton *et al*. 1997).

1.6 Mitochondrial permeability transition pore

Mitochondria play important roles in cell survival and death. In healthy cardiac myocytes they provide ATP via oxidative phosphorylation to meet the high energy demand of the heart (Halestrap *et al*. 2004). It consists of a non-specific pore known as the mitochondrial permeability transition pore (MPTP). MPTP is formed between the inner and outer mitochondrial membranes from a complex of the adenine nucleotide translocase (ANT), cyclophilin-D (CyP-D) and the voltage-dependent anion channel (VDAC) (Halestrap *et al*.,

2002). In addition, it also consists of translocator protein (TSPO) of 18kDa which was previously known as peripheral benzodiazepine receptor (Fan *et al.* 2014). TSPO is localised in the outer mitochondrial membrane and is believed to play an important role in mitochondrial cholesterol transport.

Normally, the inner mitochondrial membrane is only permeable to a few ions and metabolites. Whereas under stress, the inner membrane permeability barrier is disrupted as the MPTP opens and allows free passage of molecules of < 1.5 kDa in size (Crompton 1999). This can have deleterious effects as solutes with a small molecular weight exert a colloidal osmotic pressure by moving across the membrane, resulting in the swelling of the mitochondria and eventual outer membrane rupture releasing pro-apoptotic factors such as cytochrome c in to the inter-membrane space that play a critical role in apoptotic cell death (Halestrap *et al*. 2004).

MPTP opening also allows permeability to protons which results in uncoupling of oxidative phosphorylation (Halestrap *et al*. 2004). This causes ATP synthase to begin hydrolysing ATP which creates an energy deficit when ATP is required to fuel activity of ion pumps such as Na^{+}/Ca^{2+} exchanger to remove extra calcium and consequently leads to ATP depletion. This in turn can activate degradative enzymes such as phospholipases and proteases and also disrupt ionic and metabolic homeostasis (Halestrap *et al*. 2002). These effects can lead to irreversible cell damage and eventually result in necrotic death. Key factors responsible for the opening of the MPTP include adenine nucleotide depletion, calcium overload, oxidative stress and mitochondrial depolarization (Halestrap *et al*. 2004).

These factors are similar to those during post-ischaemic reperfusion and there is growing evidence that MPTP opening plays an important role in the transition from reversible to

irreversible reperfusion injury. Opening of the MPTP has been shown to be a critical determinant of cell death in the setting of ischaemia reperfusion injury (Hausenloy *et al*. 2003). The MPTP is considered to be open in the first few minutes of reperfusion in the setting of ischaemia reperfusion injury (Griffiths and Halestrap 1995, Di Lisa *et al*. 2001). Studies suggest that procedures including inhibition of the MPTP opening or an increase in the subsequent pore closure can reduce reperfusion injury (Halestrap *et al*. 2004). This may be either via pharmacological agents directly inhibiting MPTP opening or through an indirect effect associated with a decrease in the factors responsible for MPTP opening such as oxidative stress and calcium overload. Pharmacological inhibition of the MPTP opening has been shown to reduce myocardial injury in ischaemia reperfusion models to protect the heart. Cyclosporine-A (CSA), an immunosuppressant, and Sanglifehrin-A, have been shown to protect the myocardium from ischaemia reperfusion injury and oxidative stress (Hausenloy *et al*. 2002, Hausenloy *et al*. 2003). Clinical trials with treatment of patients with CSA for coronary thrombosis treatment have also shown improved recovery (Piot *et al*. 2008).

1.7 Biomarkers of myocardial injury

CVD is one of the leading causes of morbidity and mortality in developed countries and its prevention is a public health priority (Pearson *et al*. 2002). To aid clinical assessment in identifying patients at risk for CVD additional tools are being examined including biomarkers (Ramachandran 2006). Biomarkers help to identify high-risk individuals, accurately diagnose disease conditions and effectively prognosticate and treat patients with disease (Ramachandran 2006). Biomarkers can indicate various characteristics of a disease including genetic

susceptibility, genetic responses to particular exposures, clinical disease markers, and response to the therapy (Ramachandran 2006).

A biomarker can be easily measured in a bio-sample such as blood, urine or tissue test. It can also be obtained from a patient via a recording of, for example, blood pressure or ECG, and it may also be obtained by an imaging test such as an echocardiogram or a CT scan.

The timing of the sample collection is important as many cardiac biomarkers are known to have limited half-life (Walker 2006) and various factors including patient age, food consumption, and physical activity can alter the expression levels of traditional clinical biomarkers (Baetz and Mengeling 1971). Furthermore, many cardiac biomarkers are not specific to the heart only and their expression level may be difficult to detect (Naraoka *et al*. 2005). Some of the biomarkers that are clinically being used include troponins, creatine kinase and natriuretic peptide and will be discussed in the following section.

1.7.1 Clinical cardiac biomarkers

Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are involved in muscle contraction in skeletal and cardiac muscle (Scolletta *et al*. 2012). cTnI is cardiac muscle specific whereas cTnT is also expressed in injured skeletal muscles (Wallace *et al*. 2004). They are specific and sensitive standard biomarkers used to diagnose acute myocardial infarction and identify the risk for patients with acute coronary syndromes (Thygesen *et al*. 2007). They are released into the blood circulation and the extent of elevated serum cTnI and cTnT levels echo the magnitude of myocardial cell injury (Collinson *et al*. 2001). However, cardiac injury may not be linked with alterations of serum troponins where cardiac muscle cell membrane disruption is not altered (Jaffe *et al*. 2000, Thomas *et al*. 1999). In addition, drug-induced dysrythmias, valvular disease,

altered ion homeostasis and contractile dysfunction do not increase serum troponin levels (Wallace *et al*. 2004). Furthermore, during the first few hours of onset of myocardial infarction the troponin assays are found to be less sensitive due to their slow release from the damaged cardiac cells which usually peaks after 6-12 hours of the injury (Panteghini *et al*. 1999).

Creatine kinase isoenzyme MB (CK-MB) is also considered to mediate cardiac injury (Scolletta *et al*. 2012). CK-MB transfers the phosphate from creatine phosphate to ADP to form ATP. The MB isoenzyme form is primarily found in the myocardium, however, small quantities are also found in skeletal muscles thereby making them not a very selective indicator for myocardial injury (Antman and Braunwald 2001). CK-MB have a short time window of serum elevation and have lower specificity for cardiac tissue than troponins (Scolletta *et al*. 2012).

Another biomarker considered for the diagnosis of heart failure is the B-type natriuretic peptide (BNP) which is a hormone mainly synthesized by cardiac ventricular myocytes in response to pressure overload and volume expansion during myocardial stress or heart failure overload (Maisel *et al*. 2008, Vanderheyden *et al*. 2004). BNP release is directly correlated with the degree of ventricular wall tension (Luchner *et al*. 1998) but various studies have shown that it is not sensitive enough to be used in the diagnostic screening as its levels have been shown to vary quite considerably depending on the physiological state of the patients (Nousiainen *et al*. 2002).

1.7.2 Micro RNA

MicroRNAs (miRNAs) are a class of small non-coding RNAs that mediate post-transcriptional gene silencing (Li 2000). They are generally regarded as negative regulators of gene expression that inhibit translation and/or promote messenger RNA (mRNA) degradation by base pairing to complementary sequences within protein-coding mRNA transcripts (Kiriakidou *et al*. 2007).

This regulation is complex as one miRNA may target various mRNAs whereas one mRNA itself can be regulated by several miRNAs which can cause difficulties in comparing results from a single tissue with an entire organ/body (Thum *et al*. 2008). As miRNAs are able to regulate gene expression, they have been found to play important regulatory roles in various biological processes such as apoptosis, cell differentiation and cell proliferation (Quiat and Olson 2013).

miRNAs have been shown to be important gene expression regulators in heart development, function and cardiac pathologies which has been provided by Dicer knockout studies (Bernstein *et al.* 2003, Wienholds *et al*. 2003). Dicer is an endonuclease in the miRNA biogenesis pathway required to process miRNAs in the heart (Chen *et al*. 2008). Dicer knockout mice hearts have shown dilated cardiomyopathy and heart failure (Chen *et al*. 2008). In addition, reduced levels of Dicer protein have also been reported in human failing hearts which suggest miRNAs involvement in dilated cardiomyopathies and heart failure in human patients (Chen *et al*. 2008). Knockout of individual miRNAs such as miRNA-1 and miRNA-2 has also been shown to lead to embryonic lethality with defects in heart morphogenesis in mouse heart (Zhao *et al*. 2007).

1.7.3 miRNAs as biomarkers

Changes in miRNA expression have been found to be involved in cardiomyopathies such as acute myocardial infarction (AMI) and have been reported by various studies. Wang *et al*. (2010) showed that miRNA-208a, miRNA-499, miRNA-1 and miRNA-133a could be detected within a few hours of the onset of symptoms in plasma samples of AMI patients when compared to control subjects. In addition, miRNA-208a expression was detected at an earlier stage than cTnI and miRNA-208a was detected in all AMI patients whereas cTnI was detected in 85% of the AMI patients. Another study by Kuwabare *et al*. (2011) showed increased serum levels of

miRNA-1 and miRNA-133a in AMI patients and also that elevated levels of miRNA-133a mainly originate from the injured myocardium. Furthermore, Adachi *et al*. (2010) showed significant elevated expression levels of miRNA-499 in the AMI patients.

In addition to AMI, the expression of miRNAs has also been studied in heart failure patients by various groups. Corsten *et al*. (2010) showed a significant increase in miRNA-499 levels in acute heart failure patients. Another study by Voellenkle and colleagues (2010) investigated the miRNA expression pattern of peripheral blood mononuclear cells derived from chronic heart failure patients and showed down-regulation in the levels of miRNA-107, miRNA-139 and miRNA-142-5p (Voellenkle *et al.* 2010). Moreover, miRNA-142-3p and miRNA-29b levels were increased in heart failure patients with non-ischaemic dilated cardiomyopathy whereas miRNA-125b and miRNA-497 were decreased in patients with ischaemic cardiomyopathy (Voellenkle *et al.* 2010).

These studies clearly show the potential role of miRNAs in cardiac injury and circulating miRNAs have been proposed as biomarkers for cardiovascular diseases (Ai *et al*. 2010). miRNAs are stable in various body fluids such as blood plasma and serum (Mitchell *et al*. 2008), urine (Dimov *et al*. 2009), saliva (Michael *et al*. 2010), amniotic fluid and pleural fluid (Gilad *et al*. 2008). Secreted miRNAs are protected from degradation by ribonucleases, RNA-binding proteins and lipid vehicles (Valadi *et al*. 2007). In addition, miRNAs are specific and their levels can be easily assessed making them attractive diagnostic biomarkers (Mitchell *et al*. 2008). They can be highly effective in early diagnosis of cardiovascular diseases and their usefulness may extend to the pre-operative outcome prediction in patients undergoing cardiac surgery thereby enhancing the ability of clinicians to optimally manage patients.

Hypotheses

1) Treatment with selective mAChR antagonists, telenzepine dihydrochloride, AF-DX 116 and DAU 5884 will have detrimental effects on the heart and cause an increase in the infarct size to risk ratio in the setting of myocardial ischaemia reperfusion injury.
2) Telenzepine dihydrochloride, AF-DX 116 and DAU 5884 treatment will cause a decrease in number of viable cardiac myoctes undergoing hypoxia and reoxygenation.
3) Telenzepine dihydrochloride, AF-DX 116 and DAU 5884 treatment will result in premature opening of the MPTP under conditions of oxidative stress.
4) The natural mAChR agonist ACh and MPTP blocker CsA will protect the heart against ischaemia reperfusion injury and inhibit MPTP opening under conditions of oxidative stress.
5) Investigating the effects of individual mAChR antagonists on signalling proteins such as Akt, ERK 1/2 and SAPK/JNK will provide a better understanding of the mechanism involved in mAChR antagonist mediated injury.

Chapter Two Methods

2.1.1 Animals

Male Sprague-Dawley rats (300 ± 50 g body weight) were provided by the institutional animal house or purchased from Charles River, (Margate, UK) and had free access to standard pelleted diet and water. All procedures were carried out according to Home Office Guidance on the Operation of the Animals Act of 1986 (Stationary office, London, U.K).

2.1.2 Materials

AF-DX 116 {11-[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one}, cyclosporine A, DAU 584 hydrochloride {8-Methyl-8-azabicyclo-3-endo[3.2.1]oct-3-yl-1,4-dihydro-2-oxo-3(2*H*)-quinazolinecarboxylic acid ester hydrochloride}, ipratropium bromide {3-[3-Hydroxy-1-oxo-2-phenylpropoxy]-8-methyl-8-[1-methylethyl]-8-azoniabicyclo[3.2.1]octane bromide}, telenzepine dihydrochloride {4,9-Dihydro-3-methyl-4-[(4-methyl-1-piperazinyl)acetyl]-10*H*-thieno[3,4-*b*][1,5]benzodiazepin-10-one dihydrochloride}and acetylcholine chloride [2-(Acetyloxy)-*N*,*N*,*N*-trimethylethanaminium chloride] were purchased from Tocris Bioscience (Bristol, UK).

AF-DX 116, DAU 5884 hydrochloride and ACh chloride were dissolved in di-methyl sulfoxide (DMSO) whereas ipratropium bromide and telenzepine dihydrochloride were dissolved in water. CsA was dissolved in ethanol, ensuring that the final concentration of ethanol was less than 0.01% during the experiments (Shanmuganathan *et al*. 2005). The dissolved drugs were aliquotted and stored at -20^{0}C. All the reagents used to prepare the buffers for the Langendorff

experiments and myocyte isolations were purchased from Fisher Scientific (Loughborough, U.K).

Phospho-specific Akt (Ser_{473}), total Akt, Phospho-specific ERK1/ERK2 (Thr_{202}/Tyr_{204}), Total ERK1/2, Phospho-specific SAPK/JNK (Thr_{183}/Tyr_{185}), total SAPK/JNK, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Cell Signalling (U.K). The polyacrylamide reagents and precast gradient gels (4–15% Tris-Glycine, 4-15% acrylamide) were purchased from Bio-rad (Hemel Hempstead, UK). MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich Company (Gillingham, UK).

RNAlater and mirVana^{TM} miRNA Isolation Kit were purchased from Ambion (UK). TaqMan® MicroRNA Reverse Transcription Kit, SYBRG® Green PCR Master Mix and SYBR®Green RT-PCR Reagents Kit, U6 snRNA and miRNA specific primer sets were purchased from Life Technologies (UK). Tetramethyl rhodamine methyl ester (TMRM) was purchased from Molecular Probes (UK).

2.2 Isolated perfused heart preparation

Rats were sacrificed by cervical dislocation and the hearts were immediately placed in ice-cold Krebs Henseleit buffer (KHB) (NaCl 118.5mM, $NaHCO_3$ 25.0mM, KCl 4.8mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $CaCl_2$ 1.7mM and glucose 12mM). The hearts were cannulated and perfused on the Langendorff perfusion system at a constant pressure, and the temperature was maintained at 37.5°C. The hearts were perfused and stabilised with KHB which was oxygenated with 95% oxygen and 5% carbon dioxide (pH 7.4) (Maddock *et al.*2002). The left atrium was cut away and a latex balloon was inserted into the left ventricle and inflated up to 8-10 mmHg to measure left ventricular developed pressure (LVDP). The latex balloon was attached to a cannula connected

to a physiological pressure transducer and a bridge amp connected to a Power lab device (AD Instruments, UK). Heart rate (HR) was calculated via analysis of a one-minute period of the represented electrocardiogram trace using a Bioamp amplifier (AD Instruments, UK). Coronary flow (CF) was measured by collecting the perfusate for one minute at regular time intervals. In order to induce ischaemia a surgical needle was inserted under the left main coronary artery and passed through a small plastic tube to form a snare. Tightening the snare induced ischaemia and releasing the thread induced reperfusion. At the end of reperfusion the coronary artery was religated and perfused with 1ml of 0.25% of Evans blue in saline to delineate the viable tissue.

Figure 2.1: Ischaemic heart infused with Evans blue

At the end of the experiment the heart was weighed and frozen at -20°C overnight. The frozen hearts were transversely sliced into 2mm thick slices and incubated in triphenyl tetrazolium chloride [TTC (1% phosphate buffer)] at 37 °C for 15 minutes. The heart slices were placed into

10% formalin for 4 hours. This protocol stains the viable tissue blue, red for risk and pale for infarct areas.

The heart slices were traced onto acetate sheet using different colours for viable, risk and infarct tissue. Using an image processor and analysis program (ImageTool), total infarct area and total risk area were determined by tracing around the acetate sheet. The infarct size to risk ratio (%) was calculated by the following formula:

Infarct size to risk ratio (%) = total infarct size/total area at risk*100

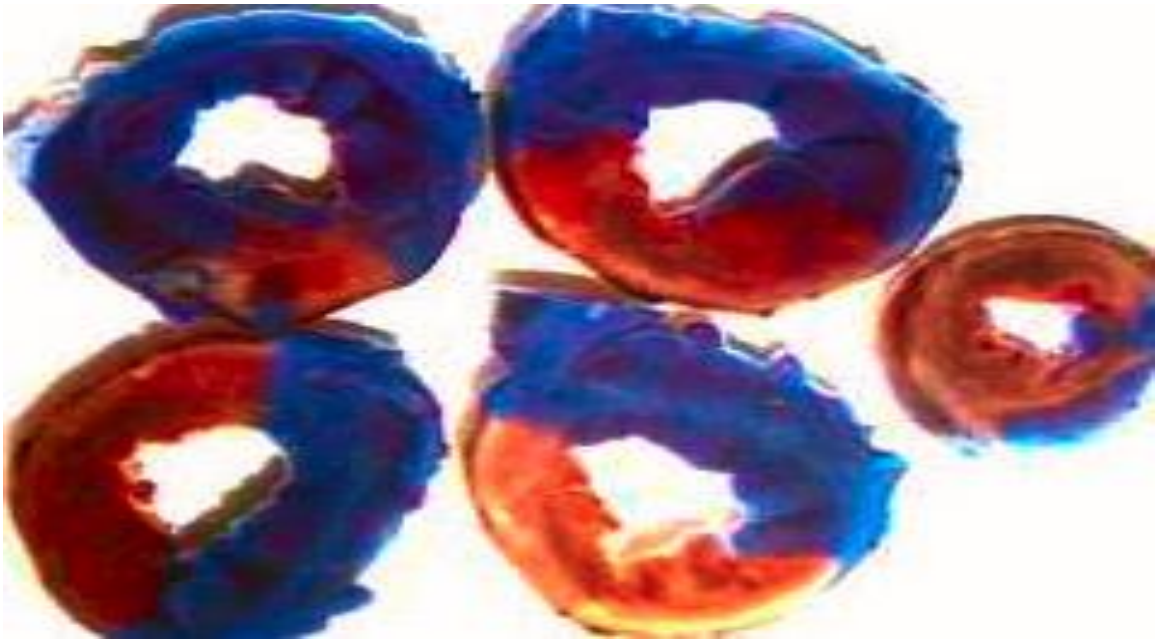

Figure 2.2: Photograph of an isolated rat heart slices stained with Evans blue. The viable tissue is stained blue, risk tissue is stained red/pink and infarct tissue is stained pale/white.

Experimental Protocol

Hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were

administered at the onset and throughout reperfusion. Figure 2.2 demonstrates the experimental protocol for Langendorff studies.

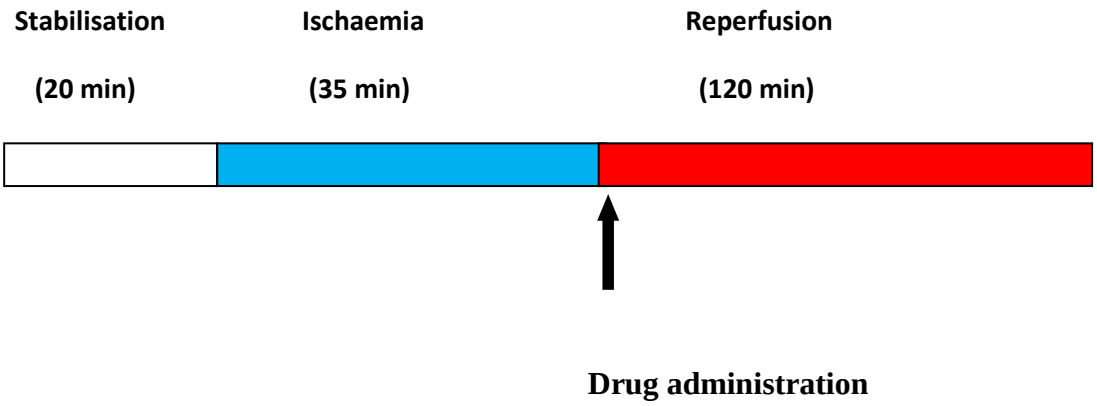

Figure 2.3: Experimental protocol for Langendorff studies

2.3.1 Isolation of cardiac myocytes

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed and the hearts were mounted on a modified constant flow (14ml/min) Langendorff apparatus and were isolated by collagenase digestion as previously described (Maddock et al. 2002). Isolated hearts were initially perfused with calcium free KHB buffer (NaCl 116.0mM, $NaHCO_3$ 25.0mM , KCl 5.4mM, $MgSO_4 \cdot 7H_2O$ 0.4mM, glucose10mM, taurine 20mM, pyruvate 5mM and $Na_2HPO_4 \cdot 12H_20$ 0.9 mM, pH = 7.4) which resulted in the cessation of heart to contraction. The buffer was oxygenated with 95 % oxygen and 5 % carbon dioxide at 37 °C. The hearts were then perfused for 5 minutes with modified Krebs Henseleit digestion buffer (0.075% Worthingtons Type II Collagenase, 4.4 μM $CaCl_2$, pH 7.4) and the effluent was collected and reused.

The atria were then trimmed away and the ventricles underwent mechanical and enzymatic digestion and incubated with fresh calcium free KH digestion buffer for 10 minutes on an orbital

shaker. The digestion buffer was aspirated and passed through a nylon mesh and the filtrate was collected and centrifuged at 400 rpm for 2 minutes. The supernatants were removed using a sterile pipette and the pellet was resuspendend in 25ml freshly prepared restoration buffer (116mM NaCl, 25.0mM NaHCO3, 5.4mM KCl, 0.4mM MgSO4 .7.H2O, 10mM glucose, 20mM taurine, 5mM pyruvate 0.9mM Na2HPO4.12H2O, 1% BSA and 1% Pen-Strep, pH 7.4).

The calcium concentration was gradually brought to 1.25mM and the cells were incubated overnight in the restoration buffer (37^{o}C, 5% CO_2). The viability of the cells was assessed by counting the number of live cardiac myocytes by visualising under a light microscope and the cell isolation yielding a viability of 65 % or more was used in the studies.

2.3.2 MTT assay

Cardiac myocytes were counted using a haemocytometer and suspended in Esumi ischaemic buffer (KCL 12nM, $MgCl_2$ 0.49mM , HEPES 4mM, deoxyglucose 10 mM, sodium lactate 20mM, 37°C and pH = 6.2) to give 1 x 10^4 cells/ml. The cells were placed in a petri dish and incubated at 37°C with conditions of 5% CO_2 <1% O_2 using a Galaxy 48R CO_2 incubator (New Brunswick, Eppendorf, Stevenage, UK). Myocytes were incubated in hypoxic conditions for 2 hours. To initiate re-oxygenation, the cells were centrifuged and re-suspended in restoration buffer. The cells were randomly assigned to drug treatment groups and placed in a 96 well plate. After 2 hours of re-oxygenation, the cells were treated with 20 µl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide (MTT) (5mg/ml in PBS) and incubated in the dark for another 2 hours. Lysis buffer (20 % SDS in 50 % N-N-dimethylformamide) was then added to all the groups and incubated at 37 °C in the dark overnight. The plate was analysed to record the

mean absorbance using an Anthos labtec instruments plate reader at 450nm. The percentage of cell viability was calculated using the relationship:

% of cell viability = mean absorbance of drug-treated sample/ mean absorbance of control *100

2.4 MPTP oxidative stress model protocol

Cardiac myocytes isolated as in 2.3.1, were plated onto laminin (Sigma) coated glass cover slips for 2 hours. The adhered myocytes were incubated in microscopy buffer (modified Krebs-Ringer's buffer with 10mM HEPES 1.2 μM $CaCl_2$) containing 3 μM tetramethylrhodamine methyl ester (TMRM) for 15 minutes. Cells were washed with microscopy buffer (KH buffer supplemented with 10mM HEPES and 1.2μM $CaCl_2$, pH 7.4) and then incubated with or without the drugs for a further 10 minutes. Plates of cells were randomly assigned to one of the drug treatment groups. To view and analyse myocytes, cover-slips were placed on the stage of a Zeiss 510 CLSM confocal microscope equipped with 20x objective lens (NA 1.3) and a heated stage. A 585-nm long pass filter allowed detection of TMRM when viewing the cells. Recording and analysis was facilitated by use of the Zeiss software package, LSM 2.8. Laser stimulation via 543-nm emission line of helium-neon (HeNe) laser was used to induce oxidative stress. Prior to laser stimulation, the cationic TMRM selectively localises in the negatively charged inner-membrane of the mitochondrion in a membrane potential-dependent manner. Laser stimulation initiates photodecomposition of TMRM thus generating mitochondrial reactive oxygen species, leading to disruption of the mitochondrial membrane and opening of the mitochondrial permeability transition pore. Depolarisation was measured as the time at which the TMRM started to become evenly distributed throughout the cell and is indicative of the initiation of the

MPTP opening. Subsequent hypercontracture of myocytes occurs shortly afterwards due to ATP depletion. The time to both depolarisation and hypercontracture were recorded.

2.5 Western blot

2.5.1 Tissue preparation

As mentioned in section 2.2, isolated rat hearts underwent ischaemia for 35 minutes and reperfusion for 20 minutes in the presence and absence of drug treatment. At the end of the experiment the hearts were stained with 0.25% Evans blue. The hearts were removed and the risk area was immediately excised and the tissue was freeze clamped in liquid nitrogen. The tissue was stored at –80°C until required.

Being cautious not to allow the tissue to thaw approximately 50 mg of the tissue was used for protein extraction by placing it in sterile Eppendorff tubes containing cold (2-4°C) lysis buffer (100mM NaCl, 10mM TRIS, 1mM ethylenediaminetetraacetic acid, (pH 8.0), 2 mM Sodium Pyrophosphate, 2 mM Sodium Fluoride, 2 mM ß-Glycerophosphate and complete protease inhibitor cocktail, (Roche, U.K) on ice. The samples were homogenised using an IKA Labortechnik T25 homogeniser. The homogenised sample was then centrifuged (Jouan HS centrifuge) at 11,000 rpm at 4°C. The supernatant containing the protein suspension was aliquoted into sterile Eppendorff tubes and the supernatant protein concentration was measured using spectrophotometry at 280nM using the NanoDrop spectrophotometer (NanoDrop Technology, Delaware, USA). 100µl of the supernatants was removed and added to newly labelled Eppendorff tubes and was diluted with 100µl of Sample Buffer (Tris 100mM (pH 6.8), DTT 200mM, SDS 2 %, Bromophenol blue 0.2 % and glycerol 20 %) followed by heating for 10

minutes at 90^{o}C and finally centrifuged at 11,000rpm for 30 seconds. Samples were stored at -20^{o}C until required.

2.5.2 Gel electrophoresis

The Bio-Rad Ready Gel precast gradient gels (4–15% Tris-Glycine, 4-15% acrylamide) (Bio-Rad, UK) were placed in the Mini-Protean II system and locked into place. The inner chamber of the 2 gels, was filled with approximately 125ml running buffer (glycine 14.42 g/l, SDS 1.0 g/l, Tris 3.0 g/l) and the combs removed. 60µg of protein containing sample buffer was loaded into each well. The outside chamber of the system was filled with approximately 400ml running buffer. Protein markers biotinylated protein ladder and dual protein ladder (Bio Rad, UK, Cell Signalling Technologies, UK) were assigned to 2 of the 12 available wells. The gel was run at 130V for 90 minutes using the PowerPac 300 (Bio-Rad, UK).

2.5.3 Transfer

After gel electrophoresis separation, the gel was transferred to The Hybond-P Polyvinyl Difluoride (PVDF) membrane. The PVDF membrane was part of a Trans-Blot Turbo transfer pack (Bio-Rad, UK). Each pack contained Whatman filter paper and a PVDF membrane all pre-soaked in transfer buffer (glycine 14.4g/L, tris 3g/L, 30% methanol). The Trans-Blot Turbo modules were assembled as per the manufactures instructions and transferred for 7 minutes at 1.3A, 25V. The Trans-Blot Turbo modules were disassembled; polyacrylamide gels discarded and PVDF membrane cut to size and placed in 15ml Blocking buffer (5% Milk in Tris Buffered Saline with 1% Tween-20 (TBS/T)) on an orbital shaker for 1 hour.

2.5.4 Antibody incubation

The membrane was then washed three times for 5 minutes in 15ml TBST. The membrane was then incubated with the antibody buffer (5% milk in TBST) with primary antibody of interest (Cell Signalling Technologies, UK) diluted 1:1000 for overnight (16 hours) on an orbital shaker. The membrane was washed three times for 5 minutes with 15ml TBST and incubated with secondary antibody diluted 1:10,000 with an anti-rabbit antibody HRP linked IgG (Cell Signalling, U.K) and HRP linked anti-biotin (Cell Signalling, U.K), for 1 hour at room temperature. The membrane was again washed three times with 15ml TBST.

2.5.5 Detection of proteins

A 1:1 mix of Super Signal West Femto (Pierce Biotechnologies, UK), an enhanced chemiluminescent (ECL), was placed on the membrane with even distribution. The membranes were exposed using a ChemiDoc XRS imager (Bio-Rad, UK), and the bands were detected and analysed using Quantity One software (v4.5.2).

2.6 miRNA analysis

Isolated left ventricular tissue was stored in RNAlater and stored at -20 ºC. The miRNA was extracted using the mirVana^{TM} miRNA Isolation Kit (Ambion, Applied Biosystems, Austin, Texas, USA) according to the manufacturer's instructions. Total RNA quantity was determined using spectrophotometry (Nanodrop Technology, Delaware, USA) and purity assessed by using the Bioanalyser RNA 6000 Nano (Agilent 2100 Bioanalyser, Agilent Technologies, UK). The absorbance was measured at 260nm and 280nm to determine the RNA integrity number (RIN)

which ensures RNA quality. Reverse transcription (RT) of RNA to cDNA was performed with MicroRNA Reverse Transcription Kit.

The cDNA was synthesised from 2000ng total RNA in a 20 μl reaction volume where RT PCR specific primers for U6 snRNA, rno-miR-1, rno-miR-27a, rno-miR-133a and rno-miR-133b were used. The PCR reaction was performed with the following setup: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 minutes.

Real time PCR was performed using a 7500 HT Real Time PCR sequence detection system (Applied Biosystems, UK) with SYBR Green PCR Master Mix with 100ng/μl cDNA synthesised above as template in a 20 μl reaction volume using the specific primers mentioned in the RT PCR reaction. The U6 snRNA was used as an internal reference gene. A non-template control was included in all experiments. The 7500 Fast Real Time PCR sequence detection software SDS version 1.4 (Applied Biosystems, UK) monitored the amplification of DNA in real time by optics and imaging system via the binding of SYBR Green fluorescent dye to double-stranded DNA. The real time PCR was performed with the following profile: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

2.7.1 Statistical analysis

All values were expressed as mean ± SEM (Standard Error of the Mean). Infarct size, cell viability, band densities, depolarisation and hypercontracture time were analysed using SPSS 12 one-way analysis of variance (ANOVA) with Fishers Protected Least Significant Difference test for multiple comparisons. Differences were considered significant at $P<0.05$. Haemodynamics were analysed using the SPSS 12 two-way ANOVA for each time point. Differences were

considered significant at P values < 0.05 and were represented as; *p<0.05, **p<0.01, ***p<0.001.

2.7.2 miRNA data calculation and analysis

Data was analysed with the comparative cycle threshold (CT) method (Sandhu 2010). The relative amount of miRNAs were calculated with the CT values of miR-1, miR-27a, miR-133a and miR-133b miRNA in relation to the CT values of U6 snRNA in the sample by the formula $X_0/R_0=2^{CTR-CTX}$, where X_0 is the original amount of target miRNA, R_0 is the original amount of U6 snRNA, *CTR* is the CT value for U6 snRNA, and *CTX* is the CT value for the target miRNA.

miRNA data was statistically analysed using SPSS 12 t-test where differences were considered significant at P-values < 0.05.

Chapter Three: Investigation into the effects of ipratropium bromide and telenzepine dihydrochloride on heart undergoing ischaemia reperfusion injury and cardiac myocytes undergoing oxidative stress

3.1 Introduction

COPD is a collective term for airway diseases such as chronic bronchitis, emphysema and chronic obstructive airways disease. It is characterised by chronic inflammation in the airways and lung parenchyma which causes the airways to become narrow (Huiart *et al*. 2005). Surprisingly, despite COPD being the fourth largest cause of mortality worldwide, studies have shown that more COPD patients in the UK and USA die from cardiovascular diseases and lung cancer rather than respiratory failure (Maclay *et al*. 2007). These statistics are supported by other evidences such as in the Towards a Revolution in COPD Health (TORCH) study, 27% of deaths amongst COPD patients were due to cardiovascular disease (Calverley *et al*. 2007). The risk factors and pathophysiology of association of COPD with cardiovascular diseases has been mentioned in section 1.1.

The non-selective M_1-M_3 antagonist, ipratropium bromide is used in the management and treatment of pulmonary conditions such as COPD (Restrepo 2007) and controversially the long-term use of such anticholinergics has been shown to increase the risk of cardiovascular related death, myocardial infarction or stroke in COPD patients with underlying heart conditions raising concerns over the safety profile of these anticholinergics (Singh *et al*. 2008, Ogale *et al*. 2010). It is therefore imperative to understand the cardiac safety of such anticholinergics at a basic science level of pre-clinical research in the setting of ischaemia reperfusion injury. This pilot study aimed to investigate the effects of ipratropium bromide in the whole heart model of ischaemia

reperfusion injury and its effects on cardiac myocytes undergoing oxidative stress via a hypoxia/re-oxygenation protocol. In addition, the effects of Telenzepine dihydrochloride, an M_1 mAchR antagonist, on myocardium in similar conditions as mentioned above were also investigated.

3.2 Methods

3.2.1 Isolated perfused heart preparation

Sprague-Dawley rats were sacrificed and the hearts were dissected as described in section 2.2. The hearts were mounted on the Langendorff system and perfused with KH buffer. LVDP, HR and CF were measured and recorded at regular intervals. The same procedure was followed as mentioned in sectioned 2.2 to calculate the percentage of infarct to risk ratio.

3.2.2 Langendorff protocol

The hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were administered at the onset and throughout reperfusion.

The hearts were randomly assigned to the following groups: a) hearts perfused with KH buffer alone without ischaemia (control); b) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion; c) hearts perfused with ipratropium bromide at a concentration range of 0.001µM-1µM; d) hearts perfused with telenzepine dihydrochloride at a concentration range of 0.001µM-1µM. The exact administration dosage of ipratropium bromide to the patient varies; but is usually administered at a range of 40µg-500µg (Boehringer Ingelheim 1987). As the bioavailability of ipratropium bromide in humans is only 7%, we used a wide concentration range of 0.001µM-1µM for ipratropium bromide and telenzepine dihydrochloride

in our study to block mAChRs at clinically relevant concentrations. Telenzepine dihydrochloride is a selective M_1 mAChR antagonist with a K_i value of 0.94 nM.

3.2.3 MTT analysis of cell viability

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed and the hearts were mounted on a Langendorff apparatus as mentioned in section 2.3.1 to isolate cardiac myocytes. The procedure mentioned in 2.3.2 was followed for the isolated cardiac myocytes to undergo hypoxia and re-oxegenation. The drugs were administered at the start of re-oxygenation. The cells were randomly assigned to the following treatment groups: a) Cells under normoxic conditions not undergoing hypoxia and re-oxygenation; b) Cells undergoing hypoxia for 2 hours and 2 hours of re-oxygenation; c) Cells treated with ipratropium bromide for 2 hours at a concentration range of 0.01µM-1µM administered at the onset of re-oxygenation following 2 hours of hypoxia; d) Cells treated with telenzepine dihydrochloride for 2 hours at a concentration range of 0.01µM-1µM administered at the onset of re-oxygenation following 2 hours of hypoxia. The % cell viability of samples was calculated as mentioned in section 2.3.2.

3.2.4 Statistical analysis

All values were expressed as mean ± SEM. Infarct size and cell viability were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Haemodynamics were assessed for statistical difference using two way ANOVA with Fishers post hoc tests using SPSS 12. Differences were considered significant at $P \leq 0.05$.

3.3 Results

3.3.1 The effects of ipratropium bromide on the infarct size from the Langendorff experiments under normoxic conditions

Whole heart Langendorff model was used to calculate the infarct size to risk ratio under normoxic conditions. The infarct size was calculated as mentioned in section 2.2. The hearts were perfused with KH buffer and ipratropium bromide (0.001µM-1µM) for 175 minutes. The results showed that the administration of ipratropium bromide had no significant effect on the infarct size as compared with the normoxic control (Figure 3.1).

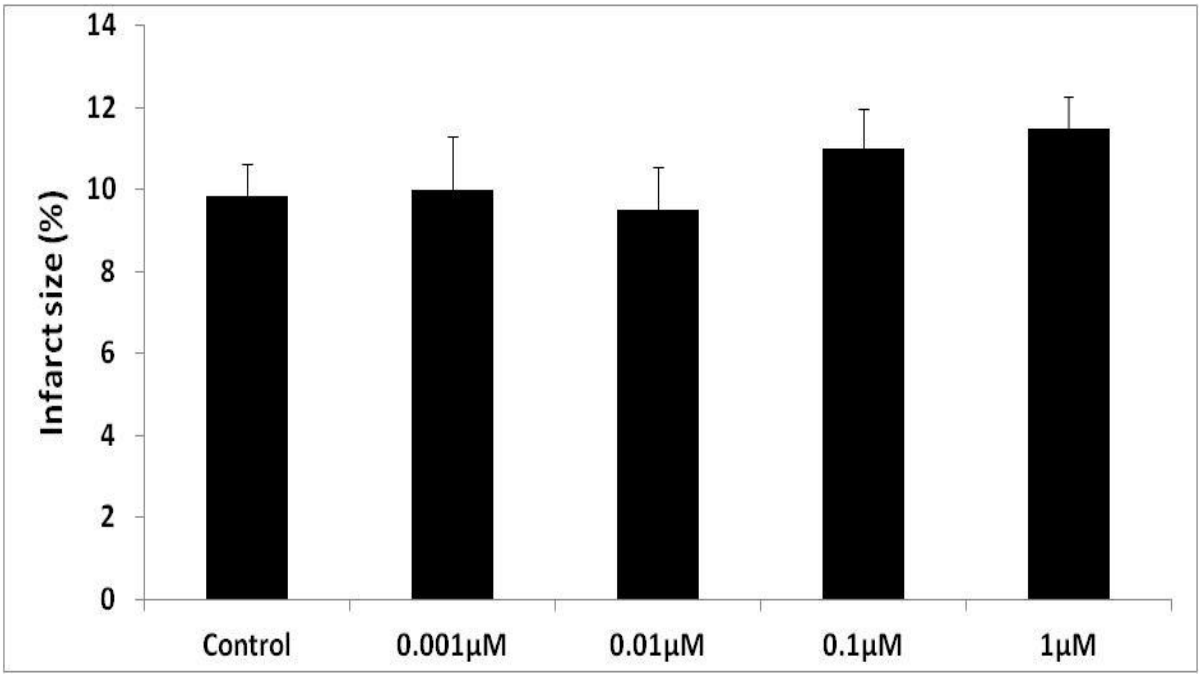

Figure 3.1: The effects of no drug treatment (control) and ipratropium bromide (0.001µM-1µM) on the infarct size as a percentage of area at risk. Results are expressed as mean ± SEM (n=6).

3.3.2 The effects of ipratropium bromide on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

The hearts were subjected to 35 minutes of ischaemia and 120 minutes of reperfusion as mentioned in section 2.2. Ipratropium bromide (0.001µM-1µM) was administered at the onset of

reperfusion. The results (figure 3.2) showed that ipratropium bromide (0.01µM-1µM) significantly increased the infarct size to risk ratio of the myocardium in a dose dependent manner as compared to the IR control [57 ± 2.1% (ipratropium, 0.01µM) vs. 47 ± 2.1% (control), $p<0.05$, 61 ± 2.49% (ipratropium, 0.1µM) vs. 47 ± 2.1 % (control), $p<0.01$, 67 ± 3.94 % (ipratropium, 1µM) vs. 47 ± 2.1 % (control), $p<0.001$]. The results are shown in Table 3.1.

Table 3.1: The effect of ipratropium bromide (0.001µM-1µM) on the infarct size to risk ratio as compared to the IR control and the relative SEM values (n=6).

Group	Control	0.001µM	0.01µM	0.1µM	1µM
Infarct size (%)	47	50.75	57	61	67
SEM	2.1	2.59	2.1	2.49	3.94

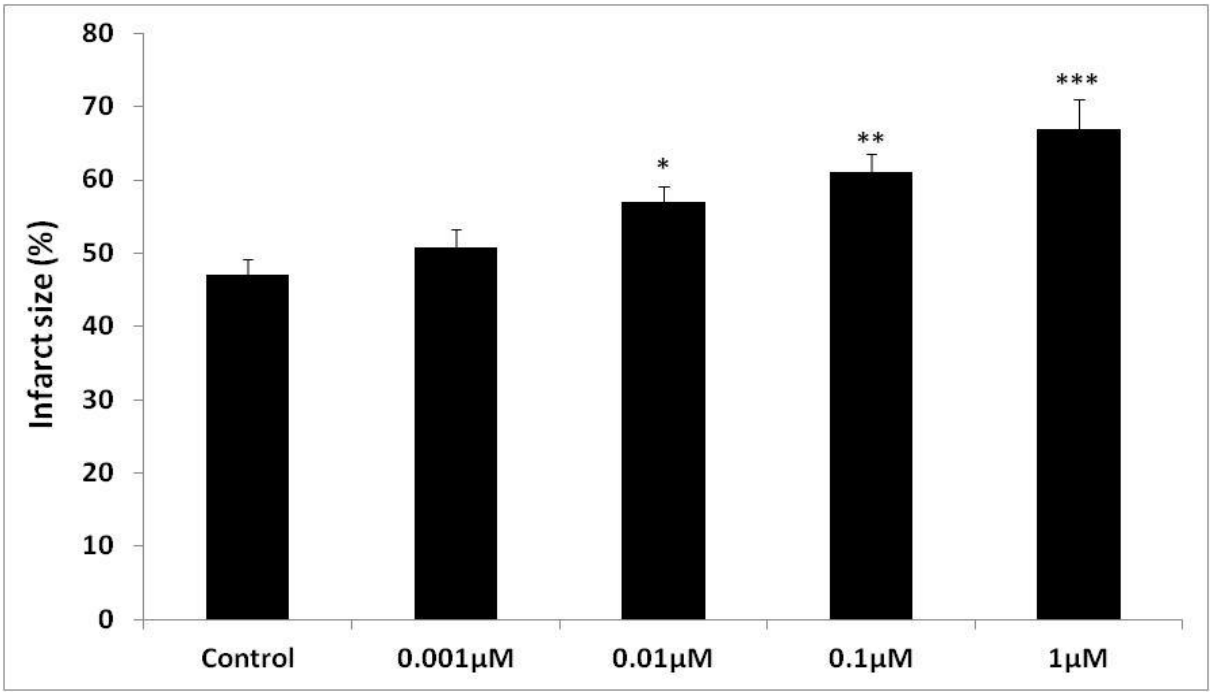

Figure 3.2: The effects of no drug treatment (IR control) and ipratropium bromide (0.001µM-1µM) on the infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01 and ***p<0.001.

3.3.3 The effects of ipratropium bromide on the viability of isolated cardiac myoctes under normoxic conditions

Cardiac myocytes were isolated following the protocol mentioned in section 2.3.1 and were incubated with MTT in the dark for 2 hours to assess the cell viability. The reduction of MTT to formazan by mitochondrial dehydrogenase and corresponding colour change was indicative of the relative changes in myocytes survival and determined the cell viability. Prior to MTT incubation, the myocytes used for the normoxic control were treated without any drug and kept under normoxic conditions for 4 hours in 6 wells of a 96 well flat-bottomed microtitre plate. The other wells were used for the myocytes being treated with the desired concentrations of ipratropium bromide (0.01µM-1µM) and incubated for 4 hours. The results (figure 3.3) showed that the cardiac myocytes being treated with ipratropium bromide (0.01µM-1µM) did not show any significant change in cell viability under normoxic conditions.

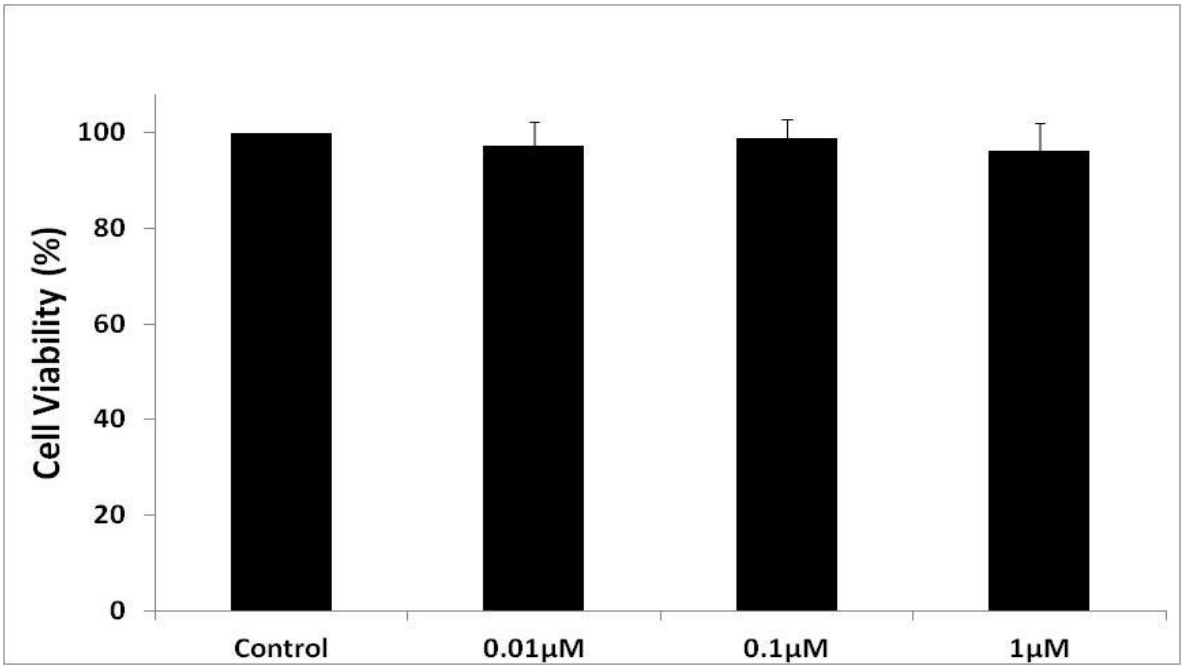

Figure 3.3: MTT analysis showing cell viability of cardiac myocytes under normoxic conditions in response to increasing concentrations of ipratropium bromide (0.01µM-1µM). Results are expressed as mean ± SEM (n=4).

3.3.4 The effects of ipratropium bromide on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

The effects of the increasing concentration of ipratropium bromide (0.01µM-1µM) on cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation were investigated. Drugs were added at the onset of re-oxygenation. As demonstrated in figure 3.4, the results showed that ipratropium bromide (1µM) resulted in a significant reduction in the number of viable cells when compared to the control i.e. myocytes not treated with drug and undergoing hypoxia and re-oxygenation (64.27 ± 5.71 % vs. 100 ± 0%, $p<0.001$).

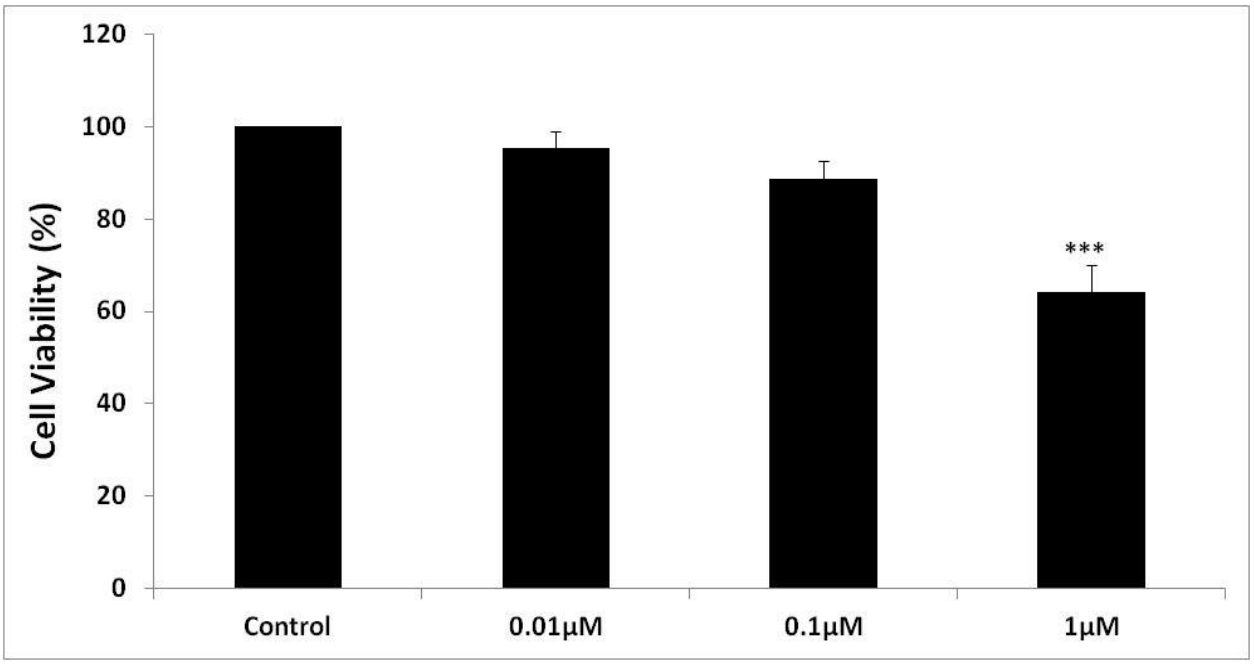

Figure 3.4: MTT analysis showing cell viability of cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation in response to increasing concentrations of ipratropium bromide (0.01µM-1µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). ***p<0.001 vs. Control.

3.3.5 The effects of ipratropium bromide on the haemodynamics of the heart

The haemodynamics including the LVDP, HR and CF of the hearts from the Langendorff model were recorded and measured. LVDP was calculated as the difference between the systolic

pressure and the diastolic pressure and presented as a percentage of mean stabilisation. The effects of ipratropium bromide (0.01µM-1µM) treatment on the LVDP are shown in figure 3.5. The results showed that the ipratropium (0.01µM-1µM) treatment did not cause a significant change in the LVDP as compared to the untreated control.

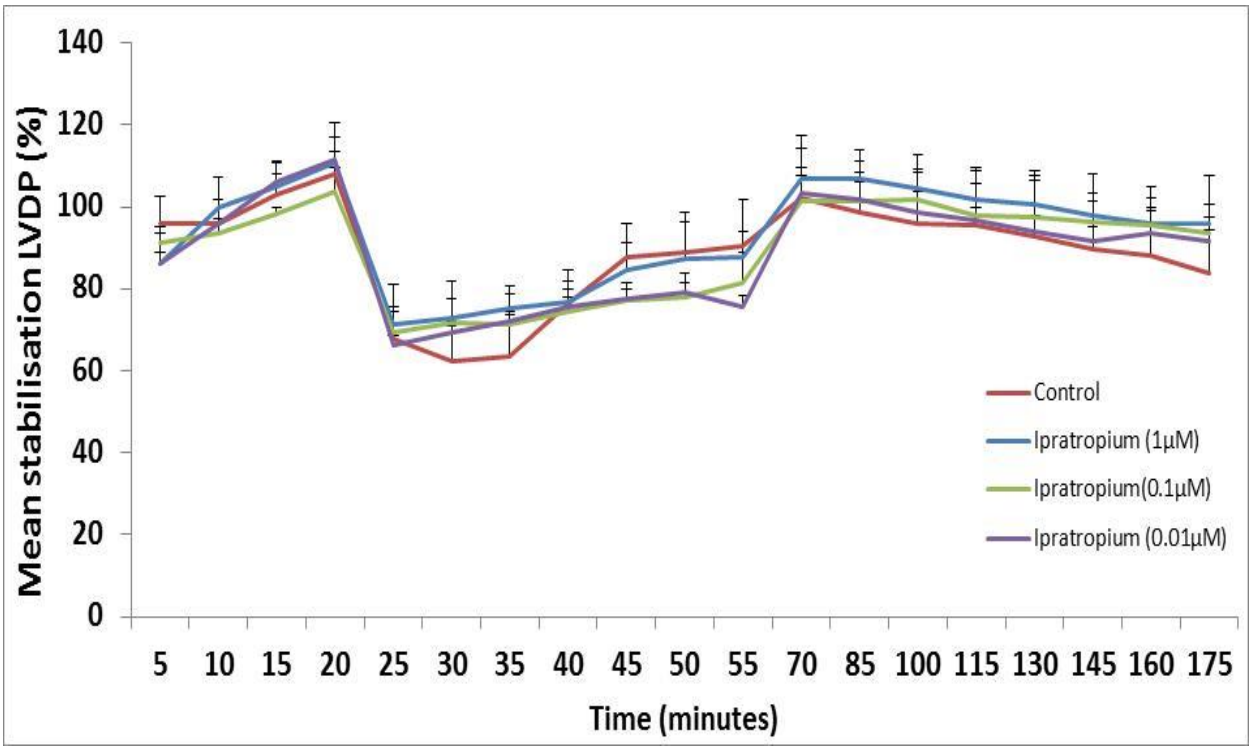

Figure 3.5: The effects of ipratropium bromide (0.01µM-1µM) on LVDP as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=6).

The effects of ipratropium bromide (0.01µM-1µM) treatment on the HR are shown in figure 3.6. The results showed that the ipratropium (0.01µM-1µM) treatment did not cause a significant change in the HR as compared to the untreated control undergoing 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion.

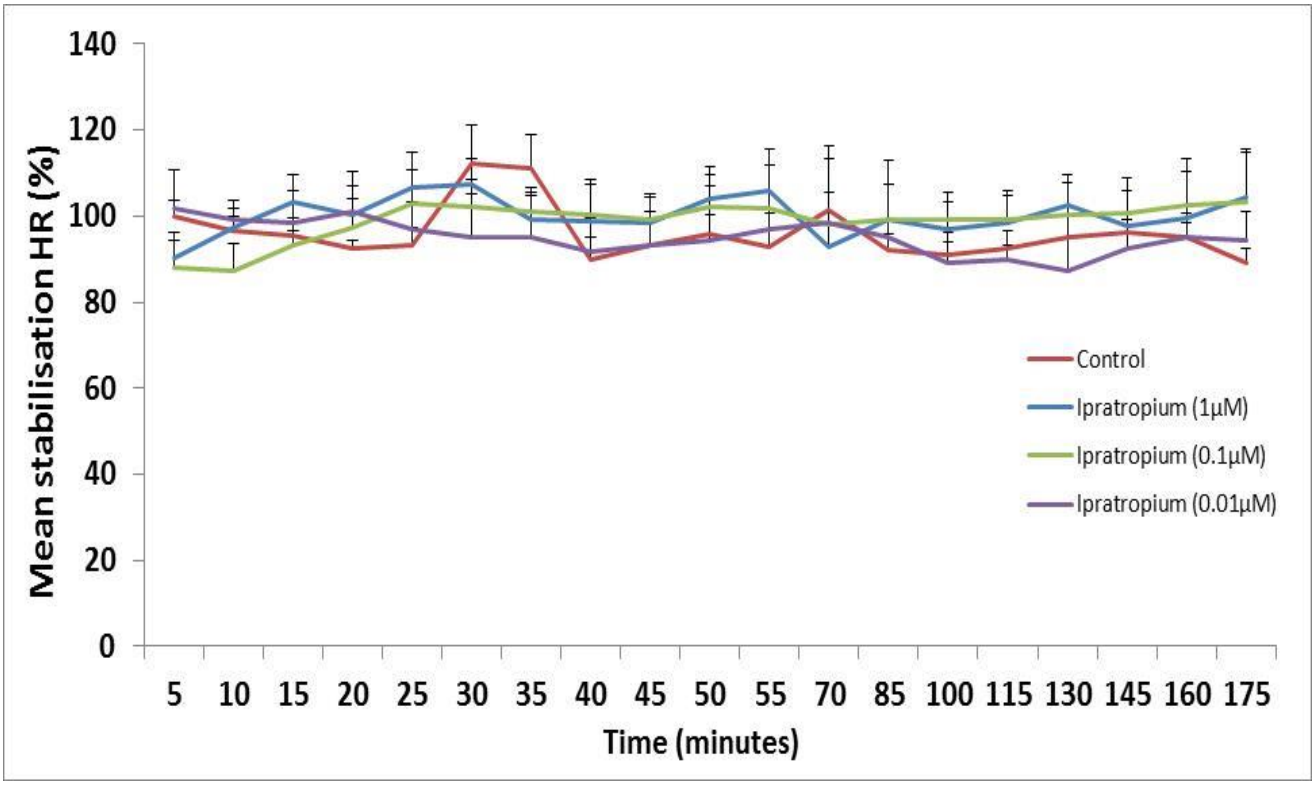

Figure 3.6: The effects of ipratropium bromide (0.01µM-1µM) on heart rate as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=6).

CF was recorded by collecting the effluent for 1 minute at regular intervals; data presented are calculated, corrected for the heart weight and plotted as a percentage of mean stabilisation. The effects of ipratropium bromide (0.01µM-1µM) treatment on the coronary flow are shown in figure 3.7. Ipratropium (0.01µM-1µM) treatment did not cause a significant change in the coronary flow as compared to the untreated control undergoing 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion.

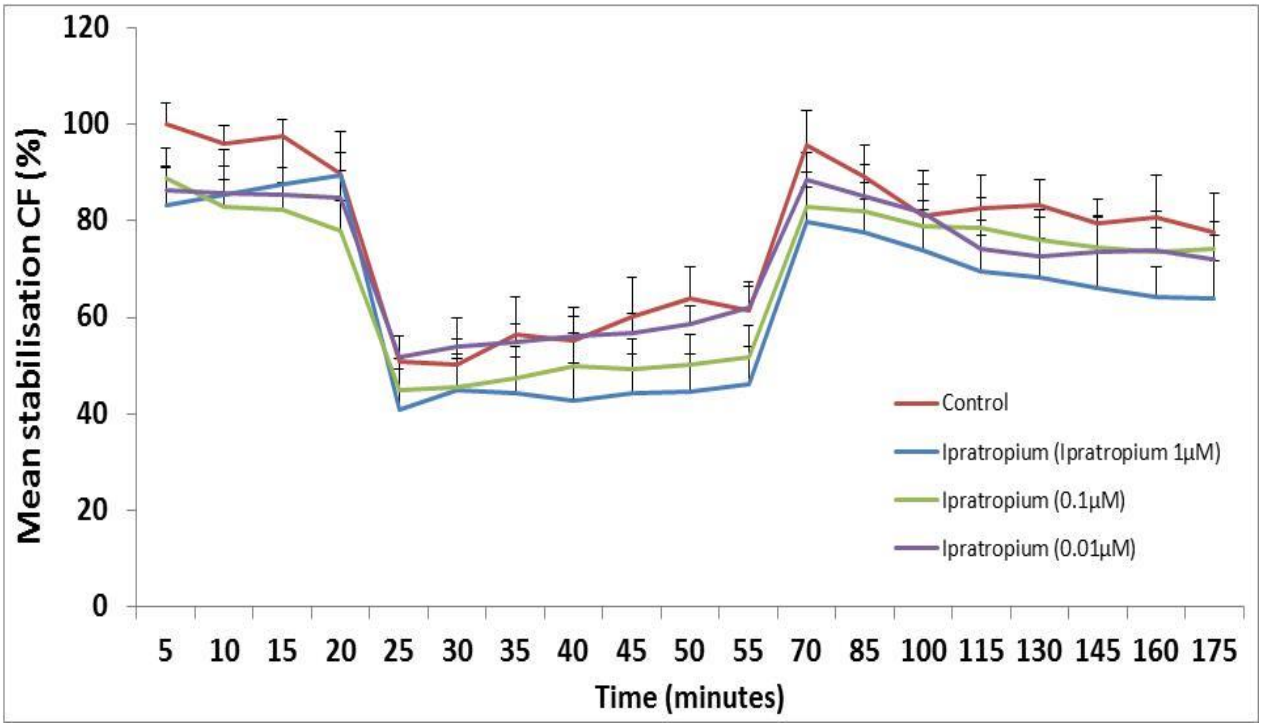

Figure 3.7: The effects of ipratropium bromide (0.01µM-1µM) on coronary flow as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=6).

3.3.6 The effects of telenzepine dihydrochloride on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

The results from the Langendorff model of ischaemia reperfusion injury (3.3.2) showed that the non-selective M_1-M_3 mAChR antagonist ipratropium significantly increased myocardial injury when subjected to the conditions of ischaemia reperfusion injury. It therefore became imperative to investigate the involvement of individual mAChR subtypes in drug mediated injury by blocking specific mAChRs. We thereby investigated the effects of a potent M_1 mAChR antagonist, telenzepine dihyrochloride on the whole heart from the Langendorff model of ischaemia reperfusion injury. The hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion with the addition of the drug at the onset of reperfusion. The results showed that the telenzepine dihydrochloride (0.001µM-1µM) did not

significantly change the infarct size to risk ratio of the myocardium as compared to the IR control as shown in figure 3.8.

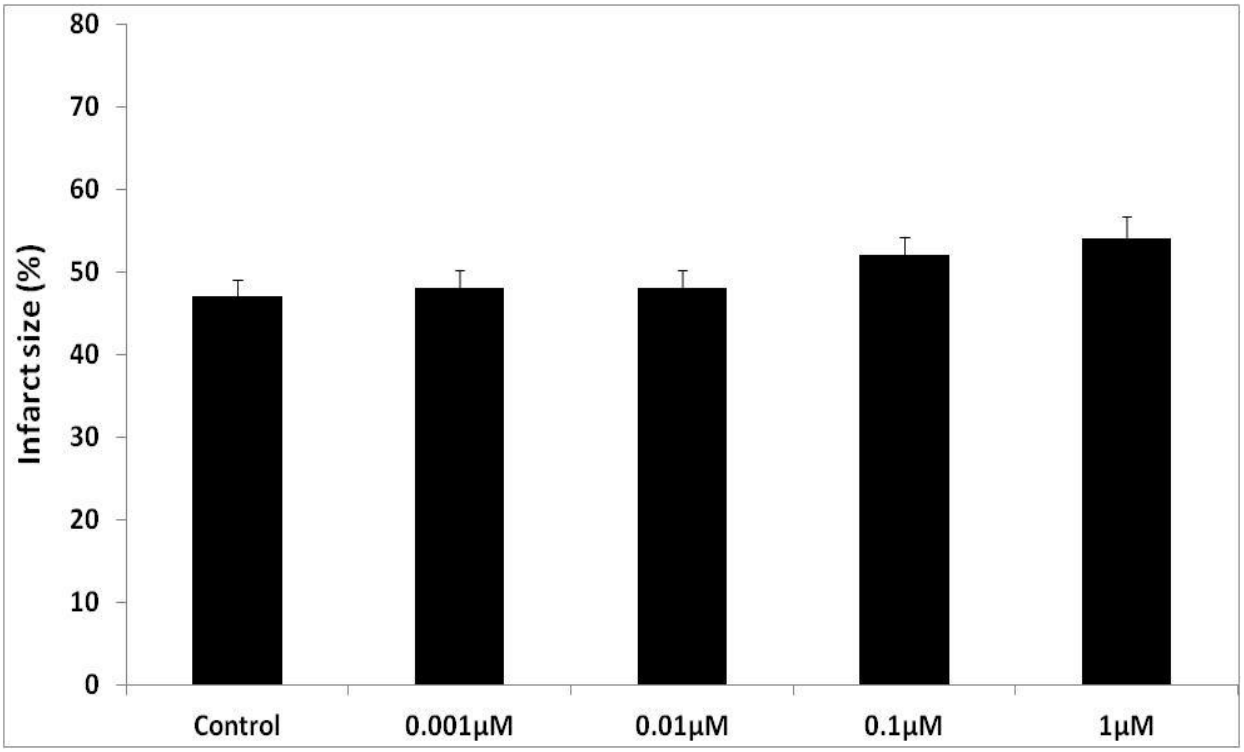

Figure 3.8: The effects of no drug treatment (IR control) and telenzepine dihydrochloride (0.001µM-1µM) on the infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6).

3.3.7 The effects of telenzepine dihydrochloride on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

The results from the cell death assay of MTT (3.3.4) showed that the non-selective M_1-M_3 mAChR antagonist ipratropium significantly decreased the cell viability of cardiac myocytes in conditions of hypoxia and re-oxygenation. We therefore investigated the effects of M_1 mAChR antagonist telenzepine dihydrochloride on isolated cardiac myocytes undergoing hypoxia and re-oxygenation. The results showed that telenzepine dihydrochloride did not cause a significant change in the cell viability of cardiac myocytes (figure 3.9).

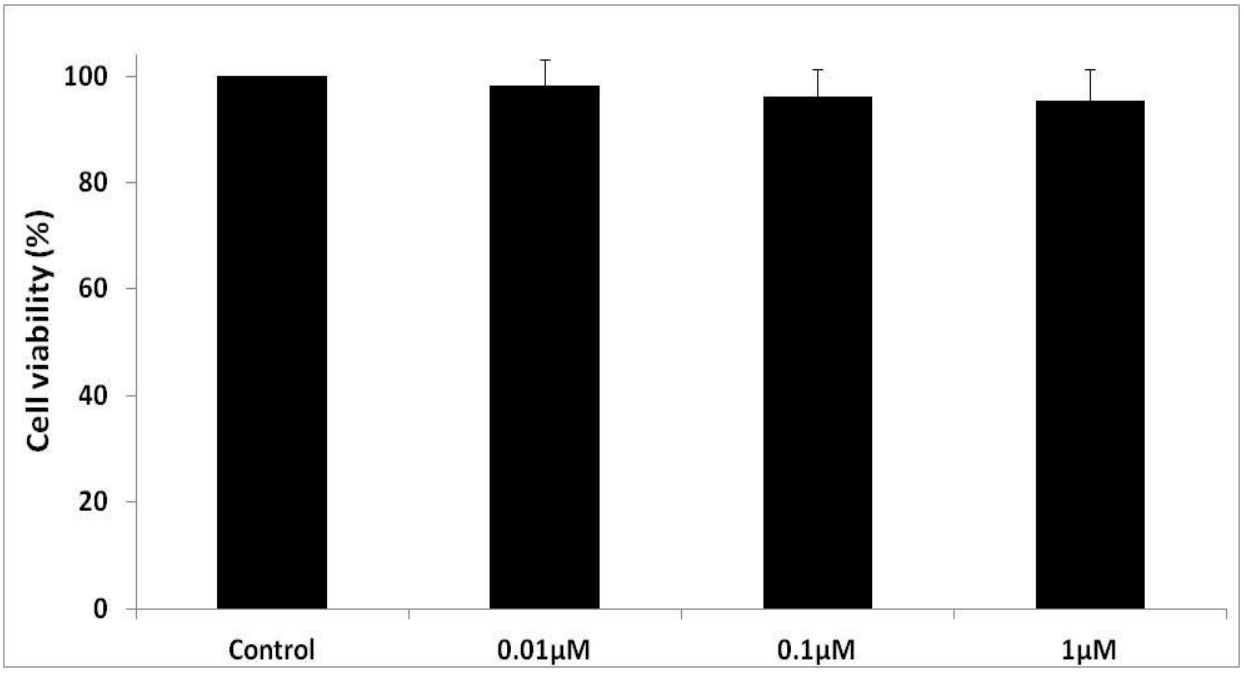

Figure 3.9: MTT analysis showing cell viability of cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation in response to increasing concentrations of telenzepine dihydrochloride (0.01µM-1µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4).

3.4 Discussion

Ipratropium bromide is a non-selective M_1-M_3 mAChR antagonist which is used as an anticholinergic bronchodilator and a widely administered therapy for COPD (Restrepo 2007). The antagonist action of ipratropium on mAChRs inhibits acetylcholine induced vagally-mediated reflexes leading to smooth muscle relaxation which facilitates airflow and alleviates bronchospasm (Restrepo 2007, Tranfa *et al*. 1995). Despite its clinical usefulness, the long-term use of this anticholinergic has been shown to increase the risk of cardiovascular death, myocardial infarction or stroke in COPD patients with underlying heart conditions raising concerns over the safety profile of these anticholinergics (Singh *et al*. 2008, Ogale *et al*. 2010). We therefore performed this pilot study to investigate the effects of ipratropium bromide on pre-clinical heart models under normoxic conditions and also in the setting of myocardial ischaemia

reperfusion injury using a Langendorff model. We also tested the effects of ipratropium on isolated cardiac myocytes using our MTT cell death assay under normoxic conditions and under oxidative stress.

The results showed that the administration of ipratropium bromide under normoxic conditions has no significant effect on the heart. The findings were supported by the results from the MTT cell death assay which also showed that ipratropium did not decrease the cell viability in the isolated cardiac myocytes under normoxic conditions. However, ipratropium bromide significantly increased the infarct size of the heart under conditions of ischaemia and reperfusion. These cardiotoxic effects were also observed using MTT cell death assay which showed that ipratropium (1µM) significantly decreased cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation. Ischaemia and reperfusion injury have previously been shown to cause inflammation in various models which may lead to tissue necrosis (Bell and Yellon 2001, De Perrot *et al.* 2003, Ng *et al.* 2005). In addition, previous studies have shown that reperfusion injury mainly occurs within the first 10-15 minutes of reperfusion which is also the time-frame for the opening of the MPTP (Halestrap *et al*. 2004, Hausenloy *et al*. 2005). Studies have also shown that ACh is endogenously released in rat heart in the absence of neuronal activity and also during ischaemia in rabbit ventricles (Brown *et al*. 1982, Kawada *et al*. 2009). As ACh is well recognised to protect the myocardium against various cellular insults including ischaemia reperfusion injury (De Sarno *et al*. 2003, Yang *et al*. 2005), we can therefore postulate that the non-selective blockade of the M_1-M_3 mAChR by ipratropium could either have a direct necrotic effect on the myocardium via inhibiting endogenous levels of ACh or could be involved in the opening of the MPTP, or both. Additional studies have been under taken in our laboratory which

revealed that ipratropium exacerbates myocardial ischaemia reperfusion injury via apoptotic and necrotic associated pathways (Harvey *et al*. 2014).

To understand the involvement of individual mAChRs in the injury implicated by ipratropium, we also investigated the effects of inhibiting the specific M_1 mAChR by telenzepine dihydrochloride in the same models. Results showed that the administration of telenzepine dihydrochloride did not have any significant effect on the heart undergoing ischaemia and reperfusion in the Langendorff studies. Furthermore, blocking the M_1 mAChR did not show a significant effect on the cell viability of the cardiac myocytes. Studies have shown that the M_2 mAChRs and not M_1 are predominantly present in the heart of various mammalian species and its activation is involved with the negative chronotropic and inotropic effects (Hulme *et al*. 1990, Caulfiled 1993). Although *in vitro* and *in vivo* studies have shown that a small population of the M_1 mAChR may exist in the mammalian heart (Leck *et al*. 1988, Brehm *et al*. 1992) their function is not entirely known.

To conclude, this pilot study has shown that the non-selective M_1-M_3 antagonist, ipratropium bromide exacerbates myocardial injury in the setting of ischaemia reperfusion injury and reduces cell viability of isolated cardiac myocytes. We have also eliminated the possibility of the involvement of the M_1 mAChR associated with cardiac injury in these models. This pilot study has laid a platform to build on to the current findings and investigate other remaining mAChRs i.e. M_2 and M_3 subtypes to clearly understand the mechanism involved in exacerbation of myocardial injury by using mAChR antagonists, such as ipratropium bromide.

Chapter Four: The M_2 muscarinic acetylcholine receptor antagonist, AF-DX 116 exacerbates myocardial injury via activation of SAPK/JNK pathway

4.1 Introduction

The mAChRs activation by acetylcholine mediates various actions in the central and peripheral nervous system (CNS and PNS, respectively) (Van Koppen and Kaiser 2003). Five distinct mAChR subtypes have been identified and characterised (M_1-M_5). The mAChRs belong to the G protein coupled receptor (GPCR) family and comprise seven transmembrane helices (TM1-7) linked by three extracellular and three intracellular loops (Lanzafame *et al*. 2003). These receptors couple to heterotrimeric guanine nucleotide-binding proteins (G proteins) and initiate signal transduction. The M_1-M_5 receptor subtypes have been pharmacologically characterised and studies have identified important physiological functions of the individual subtypes in the CNS and PNS (Hamilton *et al*.2001; Zhang *et al*. 2002).

The M_2 receptor subtype is predominantly present in the heart of various mammalian species including humans and its activation is involved with the negative chronotropic and inotropic effects (Hulme *et al*. 1990, Caulfiled 1993). The M_1, M_3 and M_5 receptor subtypes preferentially couple to the pertussis toxin-insensitive $G\alpha_{q/11}$ and $G\alpha_{13}$ subtype of G proteins leading to the activation of phospholipase C (PLC) and phospholipase D (PLD), while M_2 and M_4 subtypes preferentially couple to G_i and G_o proteins, which lead to inhibition of adenylyl cyclase (AC) (Caulfield 1993).

Muscarinic agonists and antagonists have been identified as potential novel agents for the treatment of various conditions including glaucoma, smooth-muscle disorders and gastrointestinal, respiratory and urinary diseases (Wess *et al*. 2007). However, non-selective

inhibition of multiple or all mAChRs associated with mAChR antagonists cause various side effects including dry mouth, constipation, drowsiness and blurred vision (Chapple 2000) and has limited the clinical use of these agents (Hulme *et al*. 2003).

As mentioned in the previous chapter that mAChRs such as ipratropium bromide, a non-selective M_1-M_3 antagonist is currently being used in the management and treatment of pulmonary conditions such as COPD and asthma (Restrepo 2007). Ipratropium bromide results in bronchodilation and facilitates airflow thereby alleviating exacerbations of COPD (Tranfa *et al.* 1995) and improves pulmonary function (Restrepo 2007). Despite its clinical benefits, long-term use of such anticholinergics has been shown to have adverse cardiovascular outcomes including myocardial infarction (MI), cardiovascular related death and stroke in COPD patients with underlying cardiovascular co-morbidities (Singh *et al*. 2008). According to a national study of COPD by the Healthcare Commission there are over a million individuals in the UK with COPD and over 25,000 deaths occur each year. Numerous clinical studies have indicated an increased cardiovascular risk associated with COPD patients having underlying ischaemic heart disease (IHD) that are receiving anti-cholinergic therapy (Anthonisen *et al.* 2002, Wedzicha *et al*. 2008). Although COPD in itself is responsible for systemic pathologies including IHD (Macnee *et al*. 2008), the use of mAChR antagonists by patients with underlying heart disease is suggested to further exacerbate myocardial injury. Since IHD, COPD and asthma are likely to co-exist in patients, it is imperative to assess the efficacy and toxicity of such compounds and potential beneficial effects of adjunct therapies in diseased or stressed conditions such as ischaemia-reperfusion (Gharanei *et al.* 2012). It may also be noted that people over 65 years of age are more prone to develop IHD and patients with pre-existing heart diseases are usually excluded or underrepresented in clinical trials, which aim to identify the efficacy and side effects of

treatments.

Studies have also shown that expression of specific non-coding single stranded RNA molecules called microRNAs (miRNAs) have been associated with various types of heart disease including arrhythmia, hypertrophy and heart failure (Divakaran and Mann 2008, Yang *et al*. 2007). The miRNAs have also been suggested to alter key signalling elements during ischaemia reperfusion injury. Cardiac muscle and apoptosis specific miRNAs miR-1, miR-27a, miR-133a and miR-133b have been shown to be expressed in cardiac myocytes and are involved in development of the cardiac muscle structure and heart diseases and myocardial injury (Ye *et al*. 2010, Yeh *et al*. 2012).

In the previous chapter we have already shown that ipratropium exacerbates myocardial ischaemia reperfusion injury and also reduces cell viability in isolated cardiac myocytes. Furthermore, we showed that inhibiting the M_1 mAChR by telenzepine dihydrochloride did not have any significant effect on the myocardium in the same models. As the M_2 subtype is predominantly present in the mammalian heart (Roffel *et al*. 1987), this study therefore aims to determine the effects of M_2 mAChR antagonist AF-DX 116 in the absence and presence of mAChR agonist ACh in a whole heart Langendorff model of ischaemia-reperfusion and also the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation. In addition, the effects of these compounds on cell signalling protein kinases such as p-Akt, p-ERK 1/2, and p-SAPK/JNK are investigated. The expression levels of specific cardiac miRNAs miR-1, miR-27a, miR-133a and miR-133b with the treatment of M_2 antagonist following ischaemia-reperfusion injury are also assessed.

4.2 Methods

4.2.1 Isolated perfused heart preparation

Sprague-Dawley rats were sacrificed (n=6) and the hearts were dissected as described in section in section 2.2. The hearts were mounted on the Langendorff system and perfused with KH buffer. LVDP, HR and CF were measured and recorded at regular intervals. The same procedure was followed as mentioned in sectioned 2.2 to calculate the percentage of infarct to risk ratio.

4.2.2 Langendorff protocol

The hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were administered at the onset and throughout reperfusion.

The hearts were randomly assigned to the following groups: a) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with AF-DX 116 at a concentration range of 0.001µM-3µM; c) hearts perfused with ACh (0.1µM); d) hearts perfused with co-administration of AF-DX 116 (1µM) and ACh (0.1µM). AF-DX 116 is a selective M_2 mAChR antagonist with a K_i value of 64nM for human recombinant M_2 muscarinic receptors (Hammer *et al*. 1986). ACh is the natural mAChR agonist for the M_1-M_5 subtypes (Qin *et al.* 2011).

The exact administration dosage of ipratropium bromide to the patient varies; but is usually administered at a range of 40µg-500µg (Boehringer Ingelheim 1987). As ipratropium bromide is a non-selective M_1-M_3 mAChR antagonist with bioavailability of only 7% in humans, we aimed

to use a wide concentration range of 0.001µM-3µM for AF-DX 116 in our study to block the M_2 mAChR at clinically relevant comcentrations.

4.2.3 MTT analysis of cell viability

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed (n=4) and the hearts were mounted on a Langendorff apparatus as mentioned in section 2.3.1 to isolate cardiac myocytes. The procedure mentioned in 2.3.2 was followed for the isolated cardiac myocytes to undergo hypoxia and re-oxygenation. The drugs were administered at the start of re-oxygenation. The cells were randomly assigned to the following treatment groups: a) Cells not treated with drug, and undergoing hypoxia for 2 hours and 2 hours of re-oxygenation (control); b) Cells treated with AF-DX 116 at a concentration range of 0.003µM-3µM administered at the onset of re-oxygenation following 2 hours of hypoxia; c) Cells treated with ACh (0.1µM) for 2 hours at a concentration range of 0.01µM-1µM administered at the onset of re-oxygenation following 2 hours of hypoxia; d) Cells treated with the co-administration of AF-DX 116 (1µM) with ACh (0.1µM). The % cell viability of samples was calculated as mentioned in section 2.3.2.

4.2.4 Western blot analysis of the isolated perfused heart tissue following drug treatment

Western blot analyses were carried out as described in section 2.5. The samples were randomly assigned to the following experimental groups: a) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with AF-DX 116 (1µM); c) hearts perfused with CsA (0.2µM); d) hearts perfused with co-administration of AF-DX 116 (1µM) and CsA (0.2µM). After separation, the proteins were transferred onto the

PVDF membrane and probed for the phosphorylated and the total form of Akt (Ser_{473}), Erk 1/2 (Thr_{202}/ Tyr_{204}) and SAPK/JNK (Thr_{183}/Tyr_{185}). The relative changes in the phosphorylated protein levels were calculated and corrected for differences in protein loading as established by probing for total form of Akt, ERK1/2 and SAPK/JNK 20 minutes into the reperfusion phase.

4.2.5 Evaluation of miRNA of the isolated perfused heart tissue following drug treatment

The miRNAs were isolated as described in section 2.6. Briefly, approximately 50 mg of the heart tissue that had previously been frozen in RNA*Later* was homogenised using the *mir*Vana™ miRNA Isolation kit (Ambion, Applied Biosystems, Austin, Texas, USA). The isolated RNA concentration and quality was assessed using spectrophotometry (NanoDrop Technology, Delaware, USA) and Agilent 2100 Bioanalyser (Agilent Technologies).

Following successful isolation, mature miRNAs expression patterns were quantified by reverse transcription followed by qPCR as described in section 2.6 by using the primers miR-1, miR-27a, miR-133a, and miR-133b. These miRNAs were investigated in drug treated hearts i.e. AF-DX 116 (1µM) versus the control.

4.2.6 Statistical analysis

All values were expressed as mean ± SEM. Infarct size and cell viability were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Haemodynamics were assessed for statistical difference using two way ANOVA with Fishers post hoc tests using

SPSS 12. Fold changes in the miRNA study were assessed for statistical difference using t-test. Differences were considered significant at $P \leq 0.05$.

4.3 Results

4.3.1 The effects of AF-DX 116 on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

Whole hearts undergoing ischaemia and reperfusion were used to calculate the infarct size to risk ratio from the Langendorff model. The hearts were treated with a M_2 mAChR antagonist, AF-DX 116 (0.001µM-3µM) which was added at the onset of reperfusion. The infarct size was calculated as mentioned in section 2.1. The results (figure 4.1) showed that AF-DX 116 (0.1µM-3µM) increased the infract size in a dose dependent manner as compared to the untreated control [54 ± 1.65% (AF-DX 116, 0.1µM) vs. 47 ± 2.1% (control), $p<0.05$, 56 ± 1.89% (AF-DX 116, 0.3µM) vs. 47 ± 2.1 % (control), $p<0.001$, 63 ± 1.13% (AF-DX 116, 1µM) vs. 47 ± 2.1% (control), $p<0.001$, 58 ± 1.65% (AF-DX 116, 3µM) vs. 47 ± 2.1 % (control), $p<0.001$]. The data are shown in table 4.1.

Table 4.1: The effect of AF-DX 116 (0.001µM-3µM) on the infarct size to risk ratio as compared to the IR control and the relative SEM values (n=6).

Group	Control	0.001µM	0.003µM	0.01µM	0.03µM	0.1µM	0.3µM	1µM	3µM
Infarct size (%)	47	48.5	49.7	51	52.5	54	56.1667	63.19	58
SEM	2.1	0.84	1.01	1.17	1.09	1.65	1.89	1.13	1.65

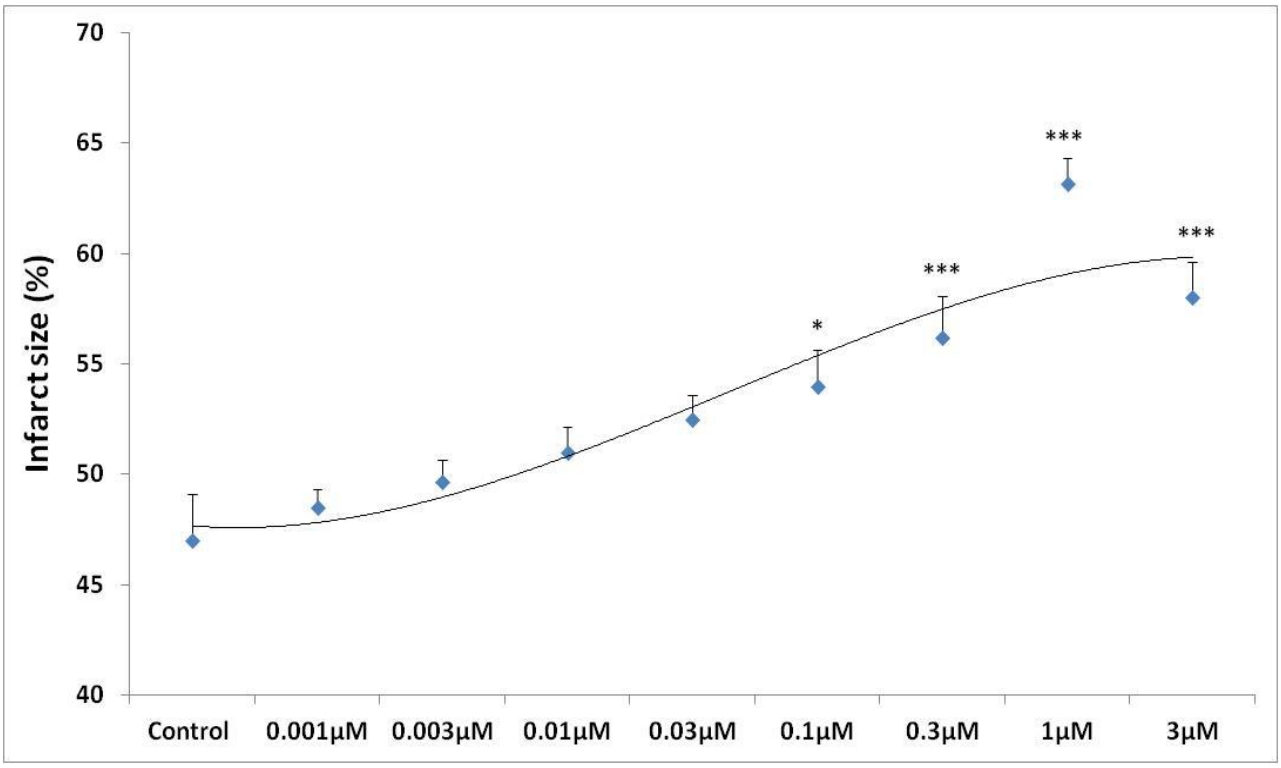

Figure 4.1: The effects of no drug treatment (IR control) and AF-DX 116 (0.001µM-3µM) on the infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). *p<0.05, and ***p<0.001 vs. Control.

4.3.2 The effects of AF-DX 116 ± ACh on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

ACh is a natural mAChR agonist that has been shown to provide protection against various cellular insults including myocardial ischaemia reperfusion injury (Yang *et al*. 2005). We therefore investigated the effects of ACh (0.1µM) and its co-administration with AF-DX 116 (1µM) on hearts undergoing ischaemia reperfuion injury. The results (figure 4.2) showed that ACh (0.1µM) significantly reduced the infarct size in comparison to the control [39 ± 2.11 % (ACh, 0.1µM) vs. 47 ± 2.09 % (control), $p<0.01$]. Interestingly, the observed increase in infarct size due to AF-DX 116 treatment (1µM) was significantly attenuated when co-administered with ACh (0.1µM) [50 ± 1.72 % (ACh, 0.1µM) vs. 63.1 ± 1.13 % (AF-DX 116, 1µM), $p<0.01$].

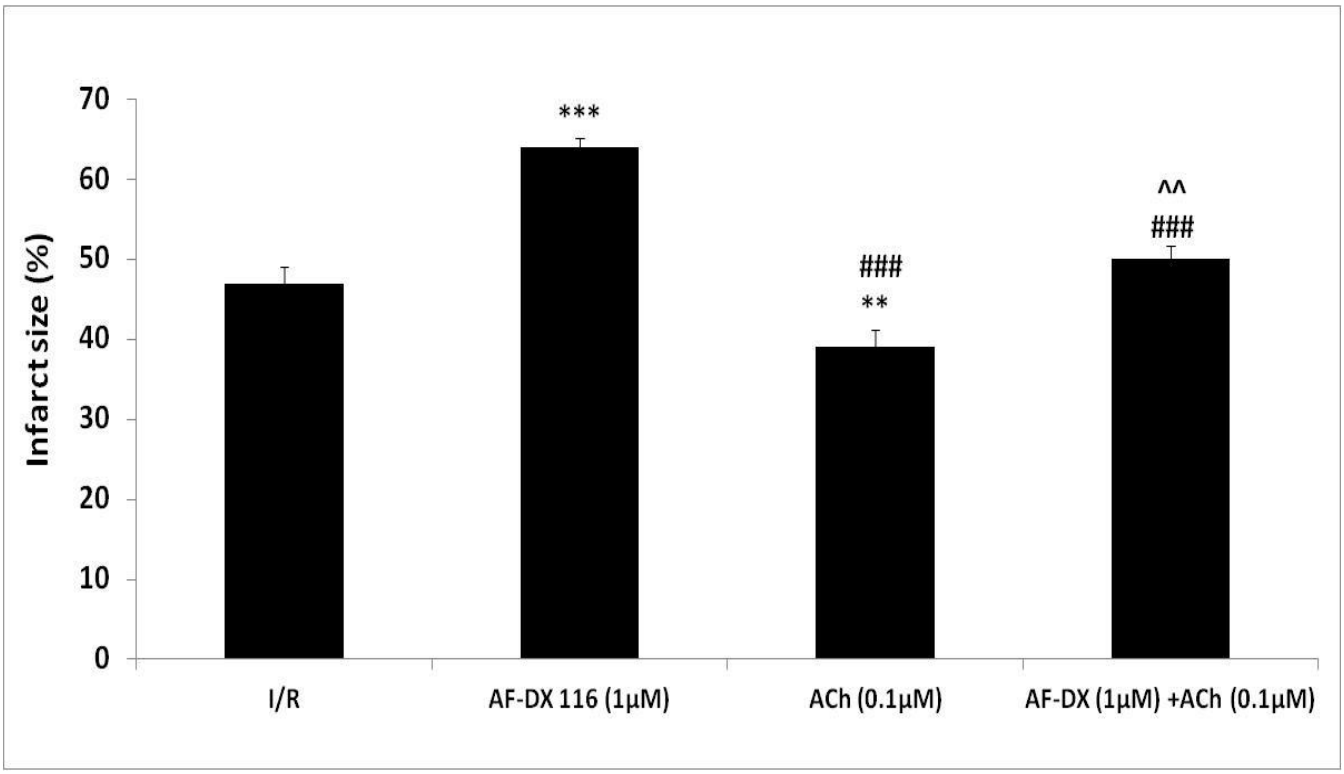

Figure 4.2: The effects of no drug treatment (control), AF-DX 116 (1µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) and ACh (0.1µM) on infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). **p<0.01 and ***p<0.001 vs. Control, ###p<0.001 vs. AF-DX 116 (1µM), and ^^p<0.01 vs. ACh (0.1µM).

4.3.3 The effects of AF-DX 116 ± ACh on the haemodynamics of the heart

The haemodynamics including the LVDP, HR and CF of the hearts from the Langendorff model were recorded and measured. LVDP was calculated as the difference between systolic pressure and the diastolic pressure and presented as a percentage of mean stabilisation. The effects of AF-DX 116 (1µM) ± ACh (0.1µM) treatment on the LVDP are shown in figure 4.3. The results showed that the AF-DX 116 (1µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the LVDP as compared to the untreated control.

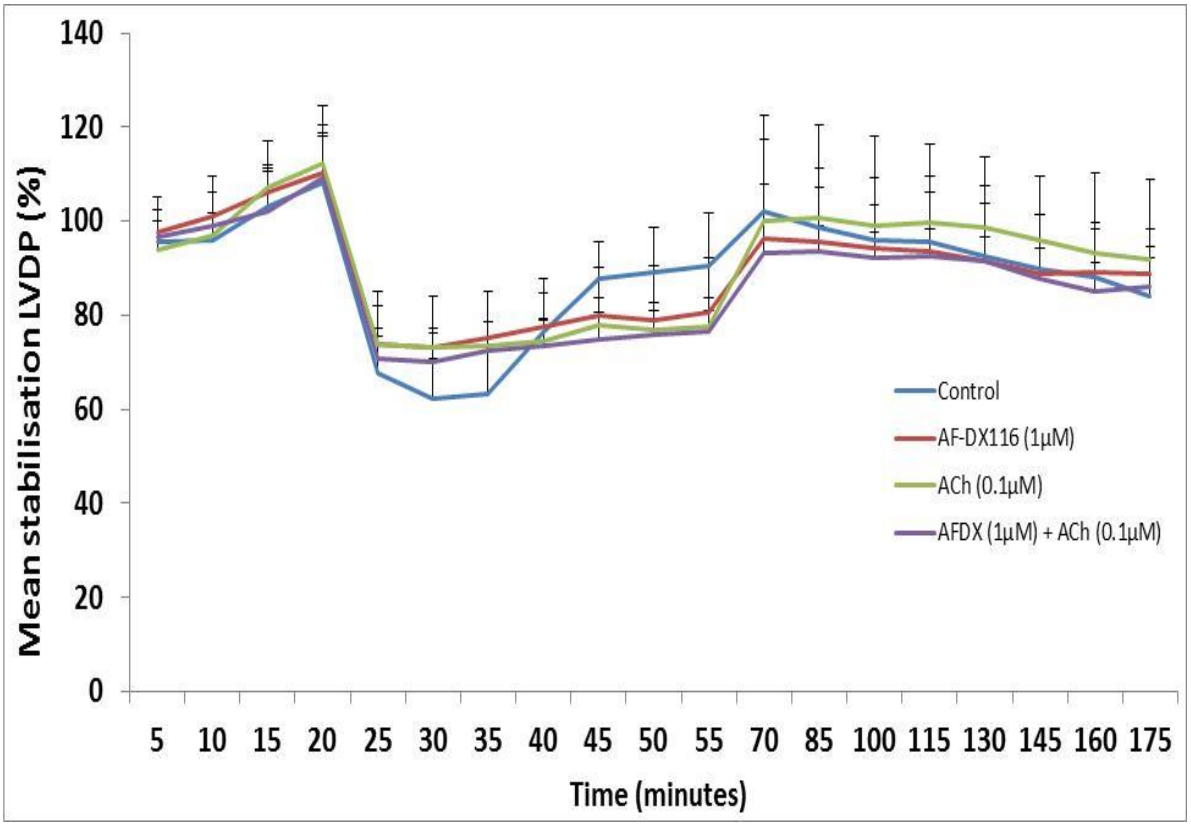

Figure 4.3: The effects of AF-DX 116 (1µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on LVDP as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

The effects of AF-DX 116 (1µM) ± ACh (0.1µM) treatment on the HR are shown in figure 4.4. The results showed that the AF-DX 116 (1µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the HR as compared to the untreated control.

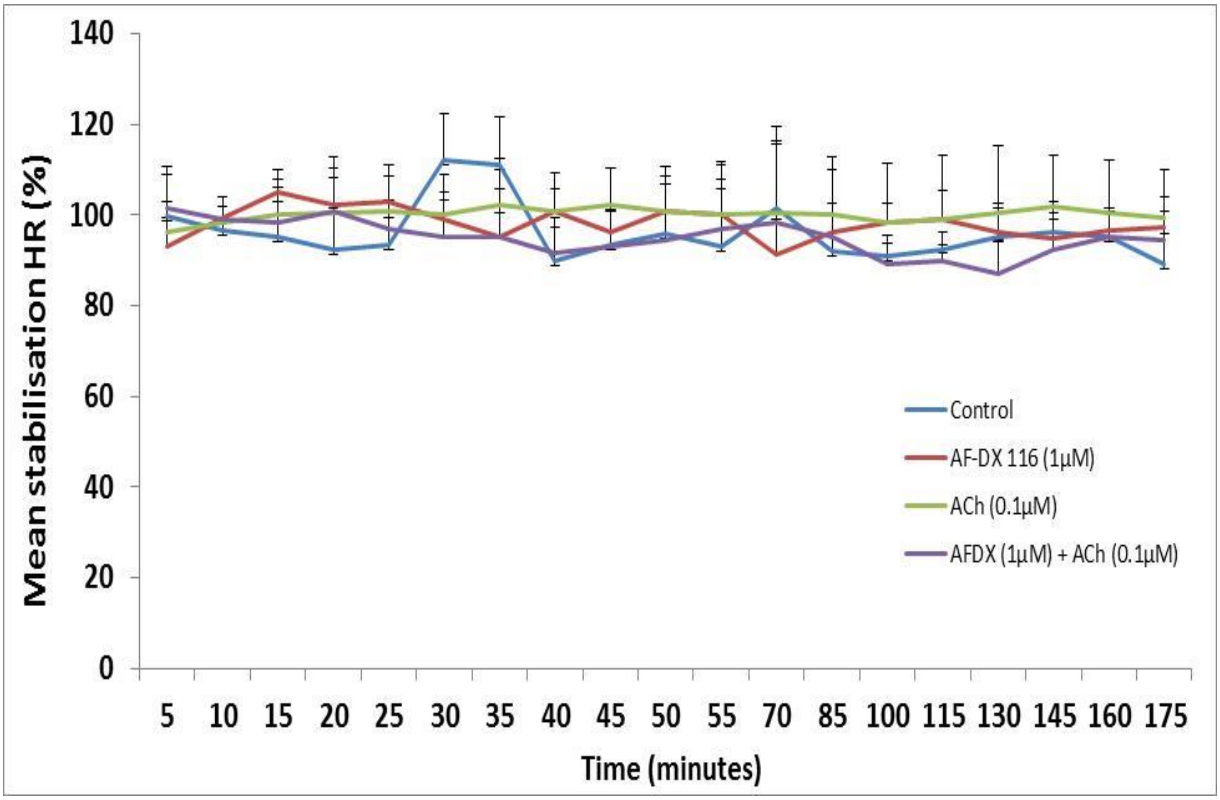

Figure 4.4: The effects of AF-DX 116 (1µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on heart rate as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

CF was recorded by collecting the effluent for 1 minute at regular intervals; data presented are calculated, corrected for the heart weight and plotted as a percentage of mean stabilisation. The effects of AF-DX 116 (1µM) ± ACh (0.1µM) treatment on the CF are shown in figure 4.5. The results showed that the AF-DX 116 (1µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the CF as compared to the untreated control.

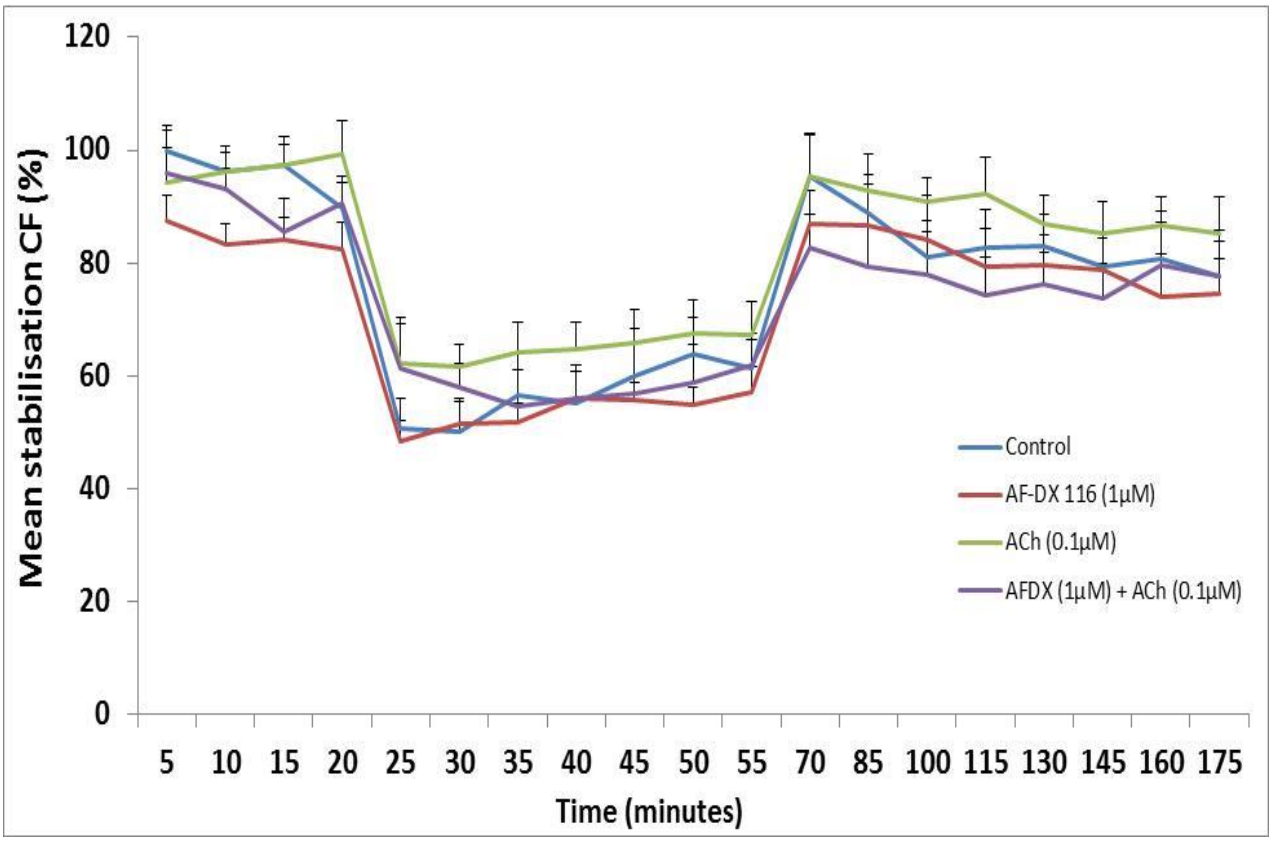

Figure 4.5: The effects of AF-DX 116 (1µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on CF as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

4.3.4 The effects of AF-DX 116 on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

Cardiac myocytes were isolated following the protocol described in section 2.3.1 and were incubated with MTT in the dark for 2 hours to assess the cell viability. The reduction of MTT to formazan by mitochondrial dehydrogenase and the corresponding colour change was indicative of the relative changes in myocyte survival and enabled to determine the cell viability. The effects of the increasing concentration of AF-DX 116 (0.03µM-3µM) on cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation were investigated which was added at the onset of re-oxygenation. The results (figure 4.6) showed that AF-DX 116 (1µM and

3µM) significantly reduced the cell viability as compared to the control [70.5 ± 6.69% (AF-DX 116, 1µM) vs. 100 ± 0% (control), $p<0.05$, 67.5 ± 5.48% (AF-DX 116, 3µM) vs. 100 ± 0 % (control), $p<0.05$]. The data are shown in table 4.2. Although AF-DX 116 was solubilised in DMSO but due to time constraint a vehicle control with DMSO was not performed.

Table 4.2: The effect of AF-DX 116 (0.003µM-3µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	0.003µM	0.01µM	0.03µM	0.1µM	0.3µM	1µM	3µM
Cell Viability (%)	100	98	95	98	82	81	70.5	67.5
SEM	0	4.64	5.63	5.2	4.04	4.98	6.69	5.48

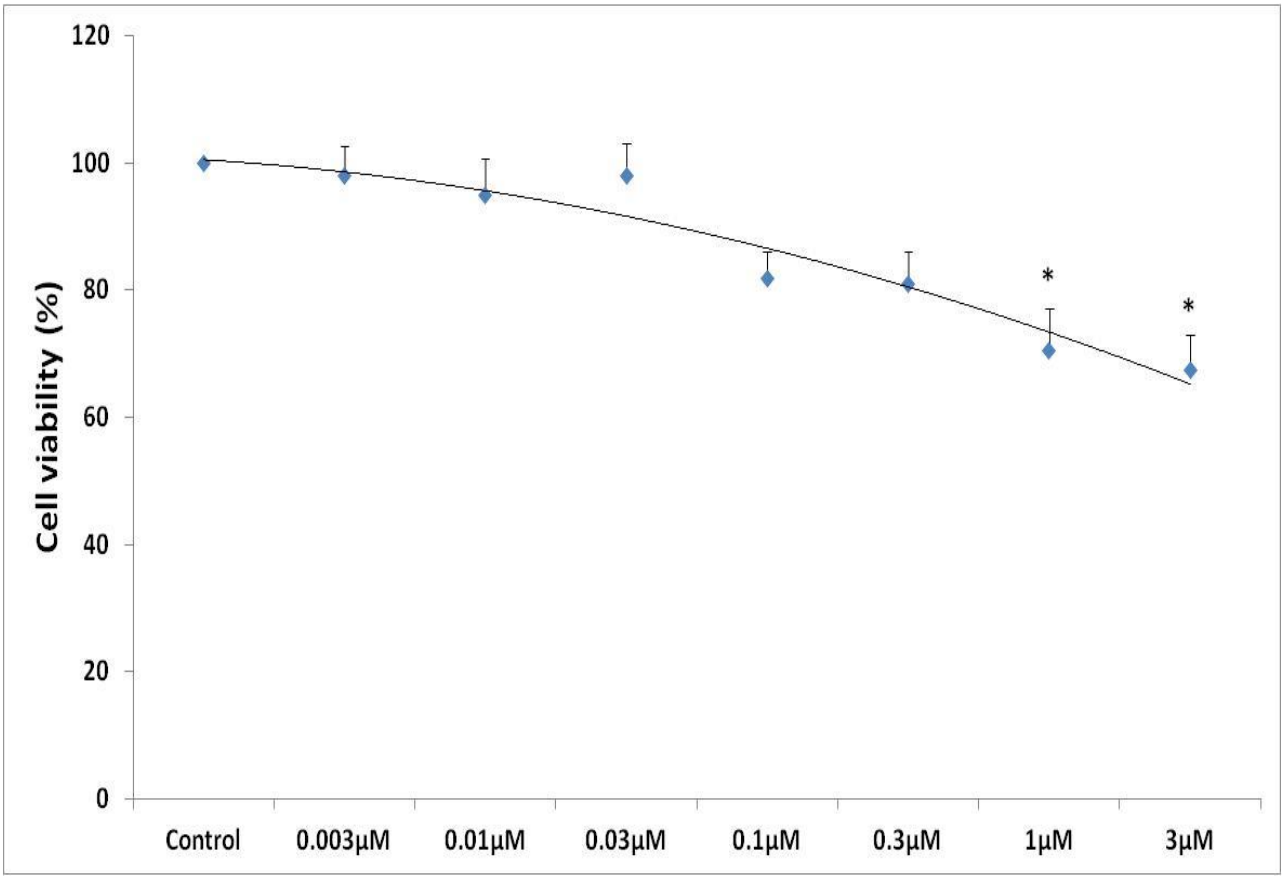

Figure 4.6: MTT analysis showing cell viability of cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation in response to increasing concentrations of AF-DX 116 (0.03µM-3µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control.

4.3.5 The effects of AF-DX 116 ± ACh on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

As the M_2 mAChR antagonist, AF-DX 116 (1µM and 3µM) was shown to decrease the cell viability of cardiac myocytes (section 4.3.4), we investigated the effects of the natural mAChR agonist, ACh on the cardiac myocytes undergoing hypoxia and re-oxygenation. The results (figure 4.7) showed that treatment with ACh (0.1µM) alone did not significantly change the viability of cardiac myocytes. However, co-administration of AF-DX 116 (1µM) with ACh (100nM) abrogated the damage caused by AF-DX 116 (1µM) [82 ± 3.55% (AF-DX 116, 1µM and ACh, 0.1µM) vs. 70.5 ± 6.69% (AF-DX 116), $p<0.05$]. The data are shown in table 4.3. Although ACh was solubilised in DMSO but due to time constraint a vehicle control was not performed.

Table 4.3: The effect of AF-DX 116 (1µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	AF-DX 116 (1µM)	ACh (0.1µM)	AFDX (1µM) + ACh (0.1µM)
Cell Viability (%)	100	70.5	92	82
SEM	0	6.69	3.16	3.55

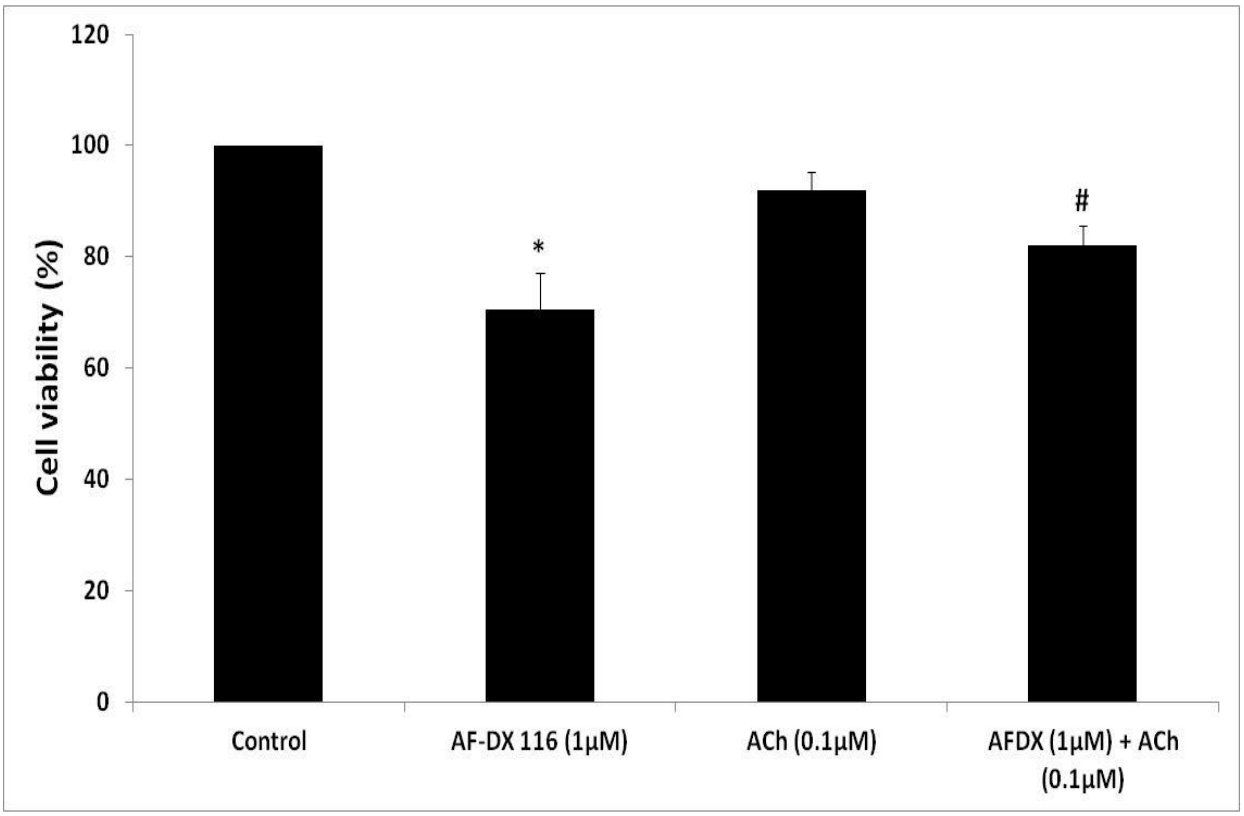

Figure 4.7: MTT analysis showing cell viability of cardiac myocytes in response to the treatment of AF-DX 116 (1µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control and #p<0.05 vs. AF-DX 116 (1µM).

4.3.6 The effects of AF-DX 116 treatment on the levels of signalling proteins as assessed by western blot analysis

To understand the molecular signalling mechanism via which AF-DX 116 has shown to exacerbate myocardial ischaemia reperfusion injury (section 4.3.1) and decreased cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation (section 4.3.4), we performed western blot analysis. The effect of drug treatment on the levels of phosphorylated Akt, ERK and SAPK/JNK at 20 minutes into the reperfusion phase was investigated.

Results showed that that AF-DX 116 (0.1µM, 1µM, 3µM) did not reveal any significant change in the levels of phosphorylated Akt as compared to the control (figure 4.8). However,

administration of ACh (0.1µM) at 20 minutes into reperfusion caused a significant increase in p-Akt levels as compared to the control [205 ± 18.5 % (ACh, 0.1µM) vs. 100 ± 0% (control), $p<0.05$].

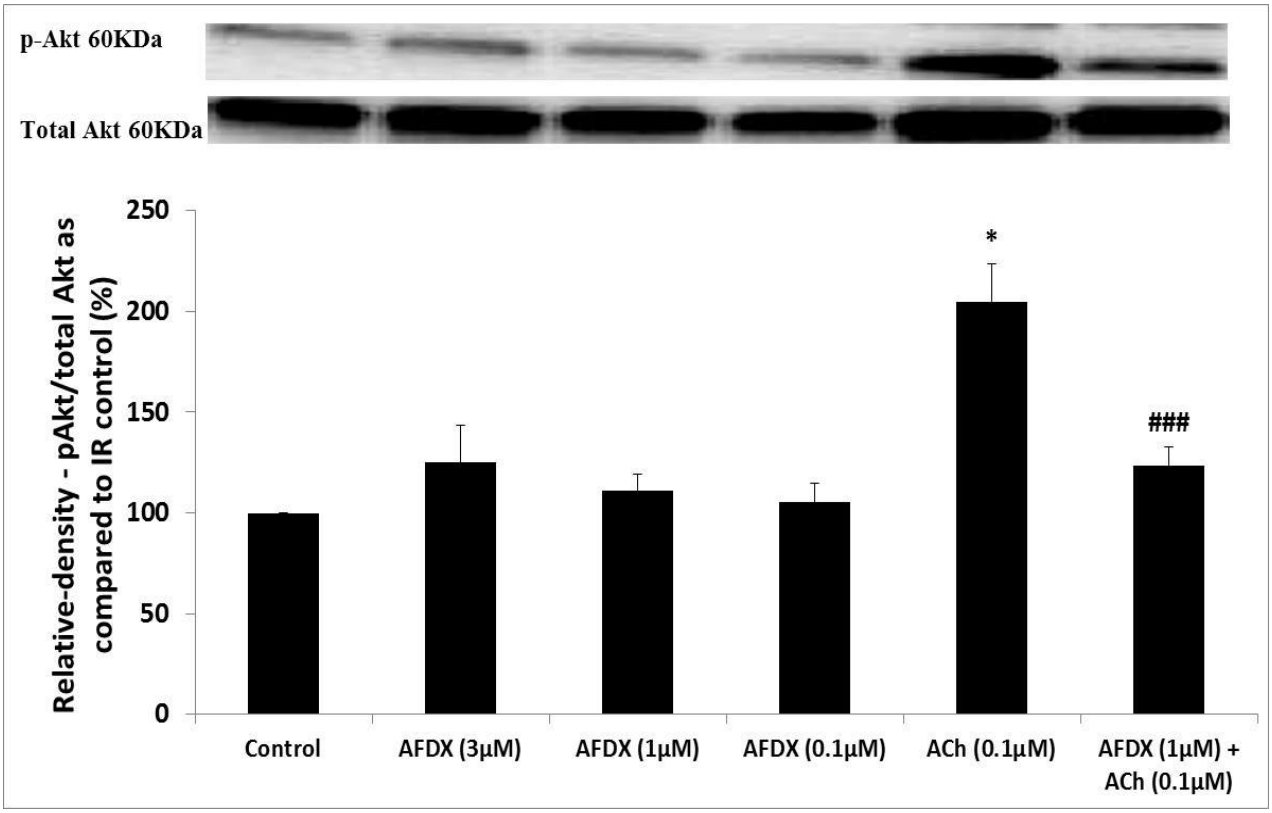

Figure 4.8: The effects of AF-DX 116 (0.1µM, 1µM, 3µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 vs. Control, ###p<0.001 vs. ACh (0.1µM).

In addition, results also showed that AF-DX 116 (1µM, 3µM) did not reveal any significant change in the levels of phosphorylated ERK 1/2 as compared to the control (figure 4.9). However, administration of ACh (0.1µM) at 20 minutes into reperfusion caused a significant increase in p-ERK 1/2 levels as compared to the control [173.8 ± 6.12 % (ACh, 0.1µM) vs. 100 ± 0 % (control), $p<0.05$].

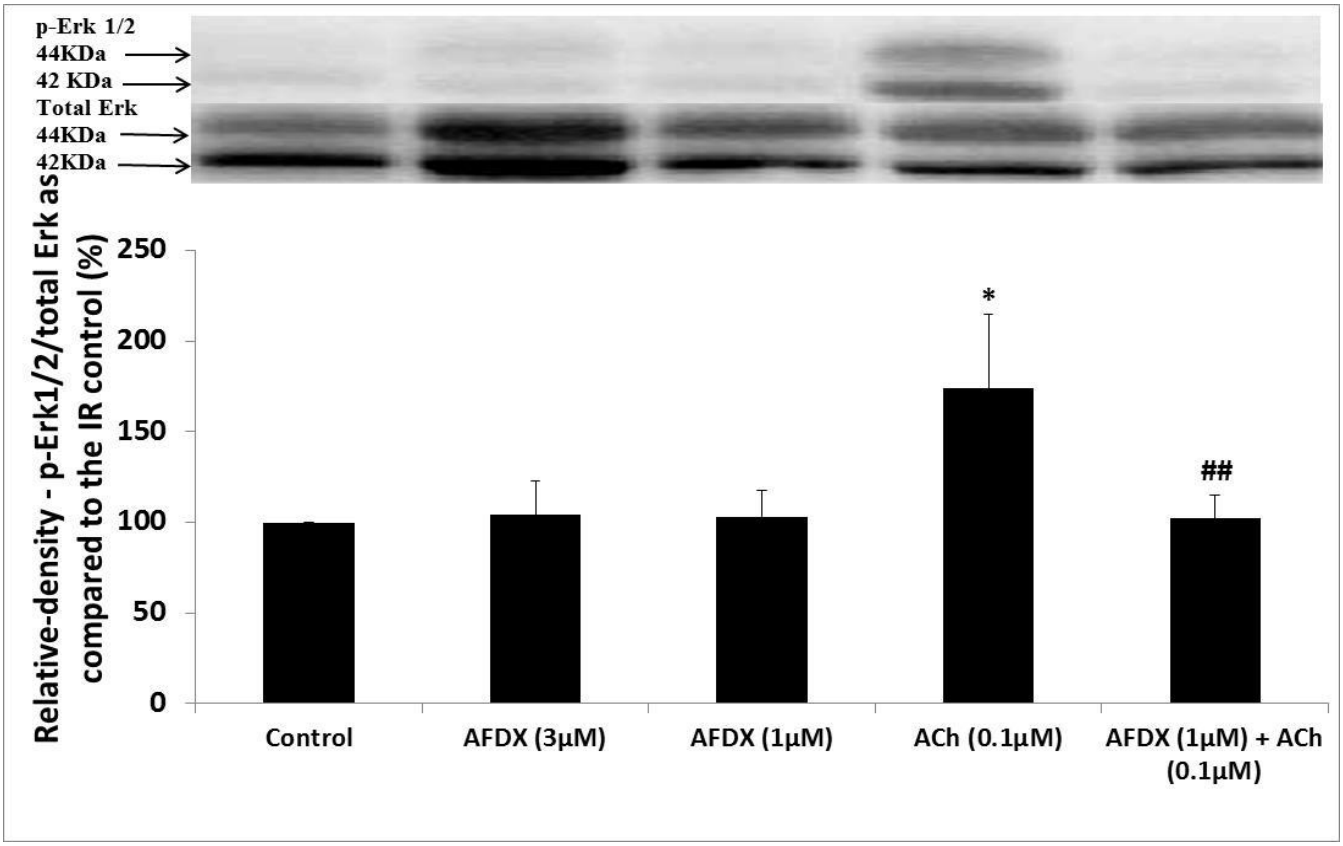

Figure 4.9: The effects of AF-DX 116 (1µM, 3µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 vs. Control, ##p<0.01 vs. ACh (0.1µM).

As represented in figure 4.10, western blot analyses also showed that AF-DX 116 (1µM, 3µM) significantly increased p-SAPK/JNK levels as compared to the control [171.9 ± 7 % (AF-DX 116, 1µM) vs. 100 ± 0 % (control), p<0.001, 160.1 ± 3.4 % (AF-DX 116, 3µM) vs. 100 ± 0 % (control), p<0.001]. In addition, ACh (0.1µM) significantly reduced p-SAPK/JNK levels as compared to the control [72.9 ± 13% (ACh, 0.1µM) vs. 100 ± 0 % (control), p<0.05]. Furthermore, the co-administration of AF-DX 116 (1µM) with ACh (0.1µM) significantly reduced p-SAPK/JNK levels as compared to the increased levels observed by AF-DX 116 (1µM) alone [132.7 ± 11.9 % (AF-DX 116, 1µM and ACh, 0.1µM) vs. 171.9 ± 7 % (AF-DX 116), p<0.05]. The data are shown in table 4.4.

Table 4.4: The effect of AF-DX 116 (0.1µM, 1µM, 3µM), ACh (0.1µM) and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	AFDX (0.1µM)	AFDX (1µM)	AFDX (3µM)	ACh (0.1µM)	AFDX (1µM) + ACh (0.1µM)
p-SAPK/JNK/Total SAPK/JNK (%)	100	123.6	171.9	160.1	72.9	132.722
SEM	0	6.12	7	3.4	13	11.9

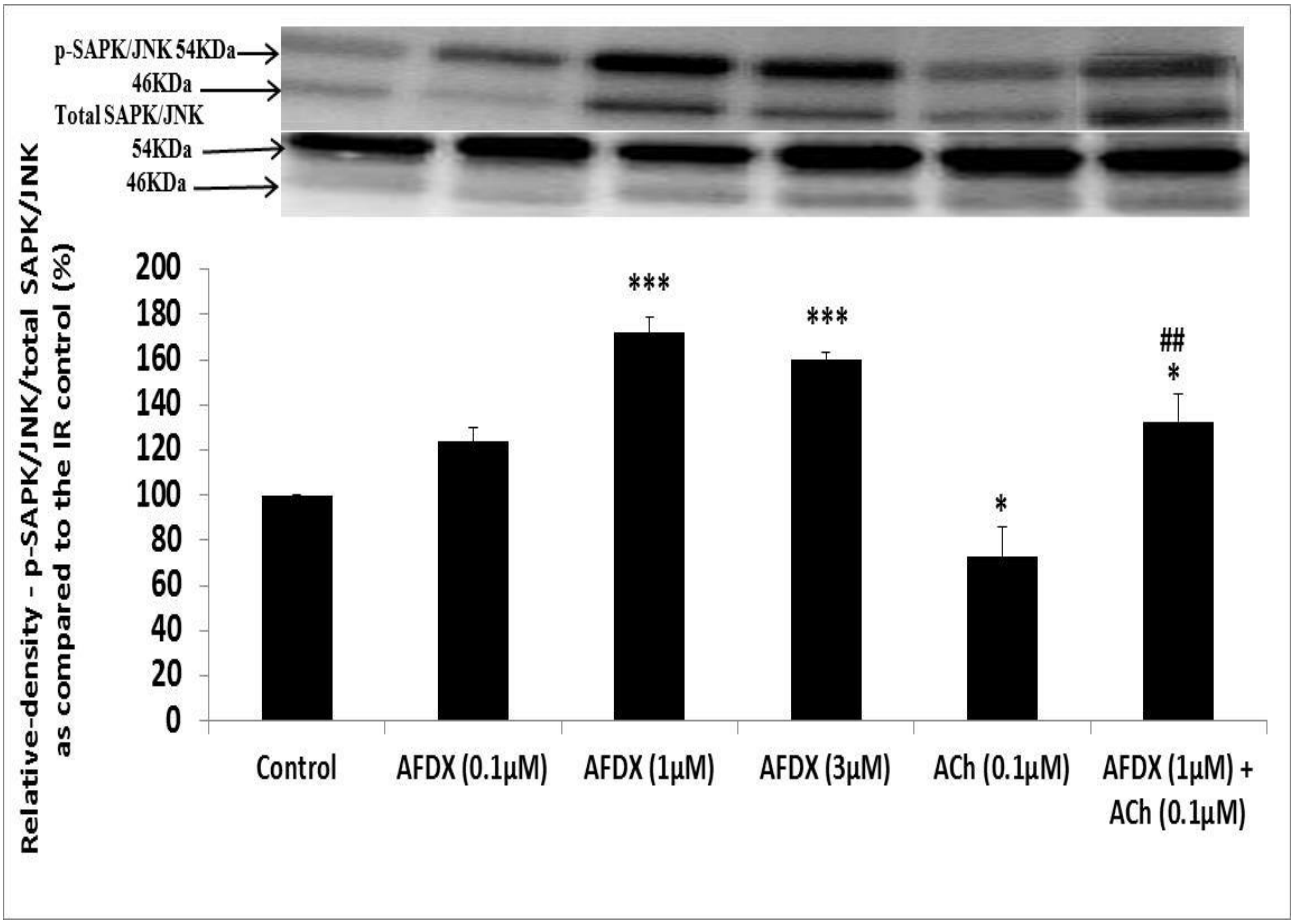

Figure 4.10: The effects of AF-DX 116 (0.1µM, 1µM, 3µM), ACh (0.1µM), and co-administration of AF-DX 116 (1µM) with ACh (0.1µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05, and ***p<0.001 vs. Control. ##p<0.01 vs.AFDX (1µM).

4.3.7 The effect of AF-DX 116 on cardiac specific microRNAs

Cardiac muscle and apoptosis specific miRNAs; miR-1, miR-27a, miR-133a and miR-133b have been shown to be expressed in cardiac myocytes and also involved in the development of the cardiac muscle structure, heart diseases and myocardial injury (Ye *et al*. 2010, Yeh *et al*. 2012). The expression levels of specific miRNAs normalised to U6 snRNA on hearts treated with AF-DX 116 were investigated. Results (figure 4.11) showed that AF-DX 116 (1µM) treatment significantly reduced the miR-1 levels as compared to the untreated control [0.11 ± 0.06 (AF-DX 116, 1µM) vs. 0.37 ± 0.1 (control), $p<0.05$, figure 4.11].

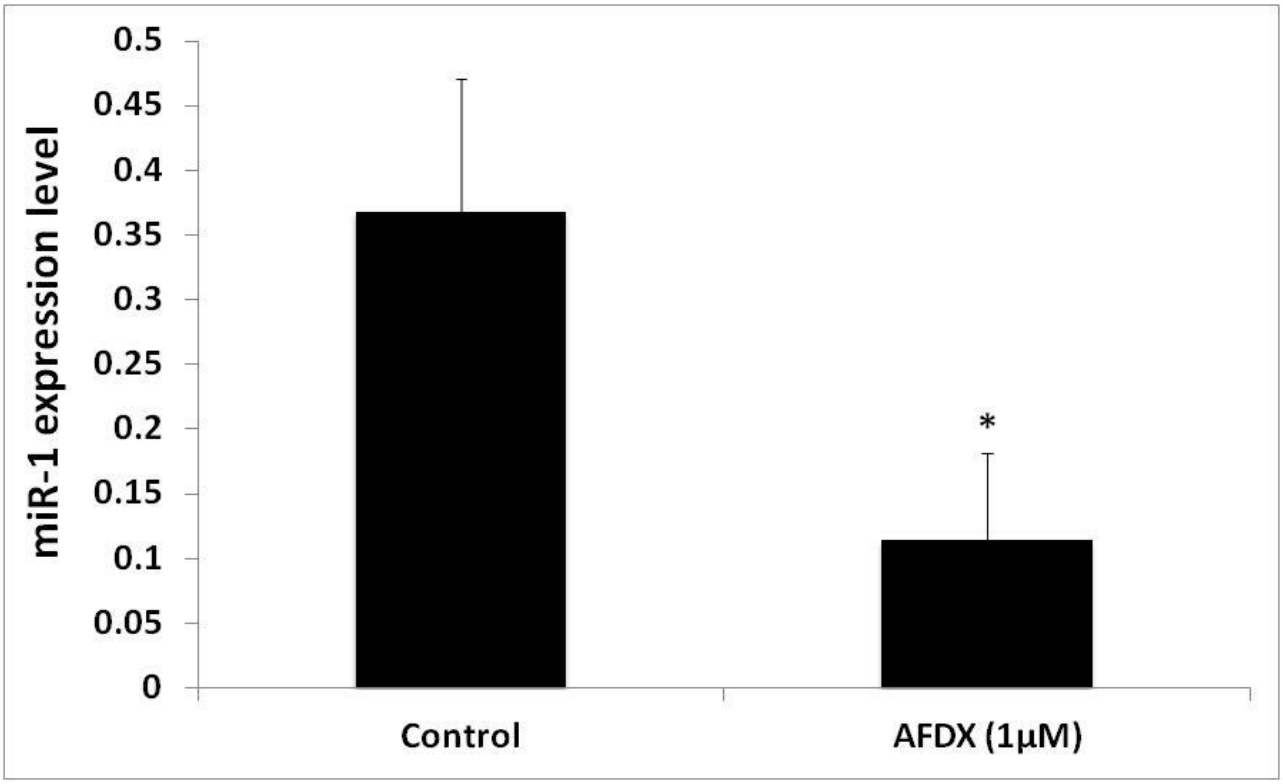

Figure 4.11: The effect of AF-DX 116 (1µM) on the expression levels of miR-1 normalised to U6 snRNA on rat heart following 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=3). *p< 0.05 vs. Control.

The results from the miRNA analysis also showed that AF-DX 116 (1µM) treatment did not lead to any significant change in expression levels of miR-27 and miR-133a (figure 4.12 and figure

4.13, respectively). However, AF-DX 116 (1µM) treatment significantly reduced the expression levels of miR133b as compared to the control [0.11 ± 0.08 (AF-DX 116, 1µM) vs. 0.28 ± 0.1 (control), $p < 0.05$, figure 4.14)].

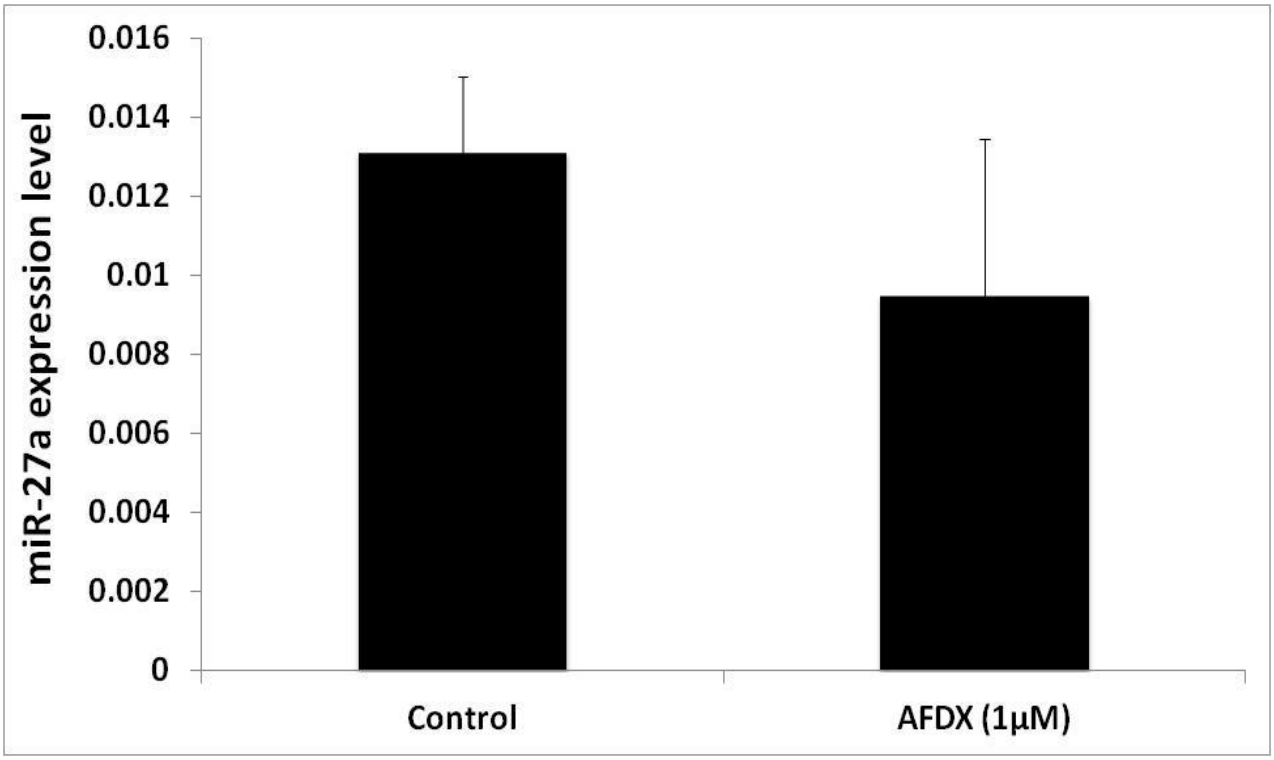

Figure 4.12: The effect of AF-DX 116 (1µM) on the expression levels of miR-27a normalised to U6 snRNA on rat heart following 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=3).

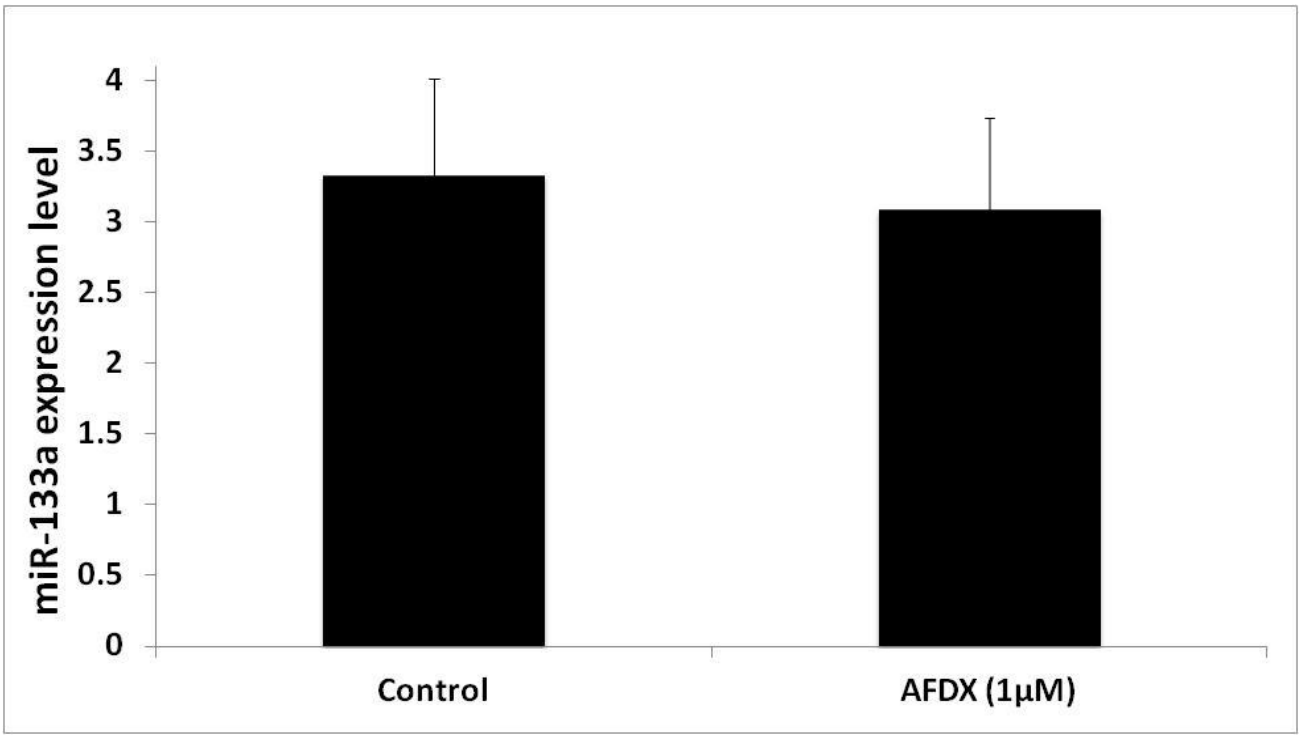

Figure 4.13: The effect of AF-DX 116 (1µM) on the expression levels of miR-133a normalised to U6 snRNA on rat heart following 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=3).

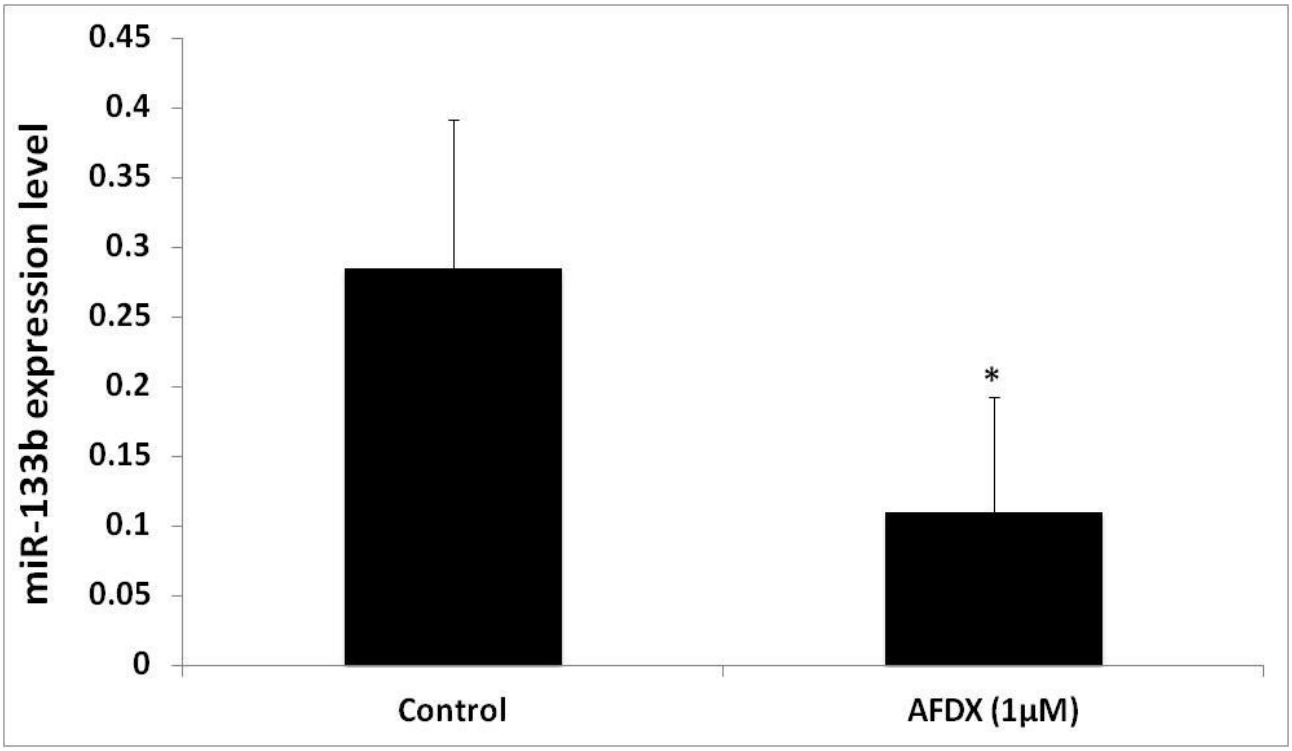

Figure 4.14: Effect of AF-DX 116 (1µM) on the expression levels of miR-133b normalised to U6 snRNA on rat heart following 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Results are expressed as mean ± SEM (n=3).

4.4 Discussion

Due to the common use of non-selective muscarinic antagonists to treat various clinical conditions and their associated side effects, it is imperative to undertake a complete cardiovascular safety profile of selective muscarinic antagonists in pre-clinical and clinical settings. The results from this chapter thereby provide essential findings which can help to start to understand the effect of M_2 muscarinic antagonism in the setting of I/R and the associated molecular mechanisms involved specifically with M_2 subtype inhibition.

The current study indicates that the M_2 mAChR antagonist AF-DX 116 significantly exacerbates myocardial injury in a dose dependent manner in *ex vivo* conditions of simulated ischaemia-reperfusion (figure 4.1). This observation was further supported by the reduction of cell viability of isolated cardiac myocytes upon AF-DX 116 treatment (figure 4.6). Furthermore, AF-DX 116 (1µM and 3µM) also activated the SAPK/JNK members of the MAPK family (figure 4.10). Studies have shown that JNK is activated in response to environmental stresses including heat shock, UV radiation, osmotic shock and inflammatory cytokines (Nishina *et al*. 2004). Myocardial ischaemia-reperfusion has also been shown to activate JNK resulting in apoptosis (Yin *et al*. 1997). Our findings confirm that blocking the M_2 subtype resulted in increased levels of SAPK/JNK, which plays integral role in cell death. Our models of ischaemia-reperfusion and hypoxia/re-oxygenation have shown to replicate the model of environmental stress to the heart and AF-DX 116 treatment has resulted in an increased phosphorylation of SAPK/JNK. To our knowledge, this is the first study to show the detrimental effects of the M_2 mAChR antagonist, AF-DX 116, in the setting of myocardial ischaemia- reperfusion injury via activating SAPK/JNK.

The differential miRNA expression profile of AF-DX 116 treatment on hearts undergoing ischaemia reperfusion injury was also investigated. miRNAs have been shown to be important gene expression regulators in heart development, function and cardiac pathologies (Bernstein *et al*. 2003, Wienholds *et al*. 2003). This has led to identification of miRNAs as biomarkers for cardiovascular diseases and myocardial injury (Ai *et al*. 2010).

miR-1 is preferentially expressed in adult cardiac myocytes and skeletal muscle and has been shown to be involved in heart disease (Ye *et al*. 2010). Bostjancic *et al*. (2010) revealed a significant down-regulation of miR-1 during myocardial infarction in humans. Our results showed that hearts treated with AF-DX 116 (1µM) during reperfusion significantly decreased the expression levels of miR-1 below the levels of the control (figure 4.11). The data therefore supports the literature from previous findings that miR-1 is down regulated in conditions of myocardial infarction.

Our results also showed that hearts treated with AF-DX 116 (1µM) during reperfusion significantly decreased the expression levels of miR-133b below the levels of the control (figure 4.14). The data supports previous studies which have shown miR-133b to be significantly down-regulated in infarcted tissue in patients with myocardial infarction as compared to healthy adult hearts (Bostjancic *et al*. 2010). Results also showed that AF-DX 116 treatment did not alter the expression levels of miR-27a and 133-a. Our findings therefore suggest that alteration in miR-1 and miR-133b expression play important regulatory roles in AF-DX 116 mediated injury. As alteration in miRNA expression in cardiac injury has also been well documented previously (Callis and Wang 2008, Kukreja *et al*. 2011), it makes them ideal potential biomarkers for cardiovascular disease and in particular for ischaemia-reperfusion injury.

In this study we also demonstrated that the mAChR agonist, ACh administered at the onset of reperfusion protected the heart from the damage caused by ischaemia- reperfusion injury and also reversed the AF-DX 116 induced damage to the myocardium (figure 4.2). The cytoprotective properties of ACh have been well documented (Critz *et al*. 2005, Li *et al*. 2011) and the resulting activation of muscarinic receptors has shown to provide protection against various cellular insults (De Sarno *et al*. 2003) including conditions of myocardial ischaemia-reperfusion (Yang *et al*. 2005). ACh has been shown to reduce the infarct size in rats during ischaemia reperfusion injury (Richard *et al*. 1995). Our results confirm previous studies that have also shown the myocardial protective effects of ACh during ischaemia reperfusion injury.

The effects of AF-DX 116 and ACh treatment on Akt and ERK signalling pathways were also investigated. A cascade of pro-survival kinases termed the RISK pathway which comprises of Akt and ERK has become an interesting target for post-conditioning. The activation of these kinases at the onset of reperfusion has been shown to reduce myocardial reperfusion injury (Hausenloy *et al*. 2005). Our results showed that treatment with ACh significantly increased Akt and ERK levels, thereby activating the RISK pathway to reduce the myocardial ischaemia reperfusion injury. Our data therefore supports the literature from previous findings that activation of Akt and ERK reduces myocardial reperfusion injury. Our results also showed that treatment with AF-DX 116 did not cause any significant change in the levels of Akt and ERK. Interestingly, co-administration of AF-DX 116 with ACh significantly inhibited the levels of Akt and ERK which were observed by treatment of ACh alone. As ACh has been previously shown to be released endogenously in rat heart in the absence of neuronal activity (Brown *et al*. 1982), AF-DX 116 could therefore be inhibiting endogenous levels of ACh acting on the M_2 subtype.

4.5 Conclusion

This is the first study to show that AF-DX 116 significantly exacerbates myocardial ischaemia-reperfusion injury via activating SAPK/JNK and down-regulating the expression levels of miR-1 and miR-133b. On the other hand, exogenous ACh has been shown to reduce this injury by activating the pro-survival kinases Akt and ERK 1/2 and down-regulating levels of SAPK/JNK. Activation of Akt and ERK 1/2 has been shown to protect the cardiac myocytes against ischaemia- reperfusion injury (Fujio *et al*. 2000) and our results confirm previous findings. Interestingly, co-administration of AF-DX116 with ACh reversed the myocardial injury. This could be because of ACh activating other mAChRs in the heart and providing a cardio-protective effect. Co-administration of AF-DX 116 with ACh reversed the myocardial injury potentially enhancing the ACh associated endogenous protective mechanism of AF-DX 116 induced injury. Further studies are therefore required for other specific mAChR subtypes in the setting of myocardial ischaemia reperfusion to determine whether they contribute to the cardiac side effects associated with muscarinic antagonists. This would help to understand the molecular mechanism of the adverse side effects for clinically used non-selective mAChR antagonists in more detail.

Chapter 5 Inhibition of the mitochondrial permeability transition pore with CsA prevents AF-DX 116-induced cardiac injury in conditions of ischaemia reperfusion injury

5.1 Introduction

Mitochondria play important regulatory roles in cell survival and death. The main function of mitochondria is to provide ATP via oxidative phosphorylation to meet high energy demands of the heart (Halestrap *et al*. 2004). However, within the mitochondria a mechanism exists which upon activation actively induce apoptotic and necrotic cell death. This is mediated by the opening of the MPTP which remains closed under normal physiological conditions but can open under cellular stress (Halestrap *et al*. 2004). MPTP is formed between the inner and outer mitochondrial membranes from a complex of the ANT, Cyp-D and the VDAC.

Under normal physiological conditions, the inner mitochondrial membrane is impermeable to all but a few ions and metabolites, but in stress, MPTP can open allowing free passage of molecules < 1.5 kDa in size, disrupting the permeability barrier of the inner membrane (Crompton 1999). This can have deleterious effects as small molecular weight solutes can move across the membrane freely, exerting a colloidal osmotic pressure that causes swelling of the mitochondria and eventual rupture of the outer membrane, releasing pro-apoptotic factors such as cytochrome c to the inter-membrane space (Halestrap *et al*. 2004). Opening of the MPTP also allows permeability to protons, which results in uncoupling of oxidative phosphorylation and, consequently, ATP depletion. This in turn can disrupt ionic and metabolic homeostasis and activate degradative enzymes such as phospholipases, nucleases and proteases (Halestrap *et al*. 2002). These changes can cause irreversible damage to the cell and eventually result in necrotic death. In addition, reperfusion results in the generation of ROS which can interact with and

damage various mitochondrial proteins including electron transfer chain components, and cause lipid peroxidation (Kroemer 2003).

The opening of the MPTP is a critical determinant of cell death in the setting of ischaemia-reperfusion injury (Crompton *et al*. 1999). It is believed to open in the first few minutes of reperfusion (Griffiths and Halestrap 1995). The factors responsible for increasing its opening include ATP depletion, oxidant stress, and high mitochondrial calcium and inorganic phosphate load (Hausenloy *et al*. 2003). These factors are similar as those during post-ischaemic reperfusion and there is growing evidence that MPTP opening plays an important role in the transition from reversible to irreversible reperfusion injury. Opening of the MPTP has been shown to be a critical determinant of cell death in the setting of ischaemia reperfusion injury (Hausenloy *et al*. 2003).

Studies suggest that procedures including inhibition of the MPTP opening or an increase in subsequent pore closure reduce reperfusion injury (Halestrap *et al*. 2004). This may be either via pharmacological agents directly inhibiting MPTP opening or through an indirect effect associated with a decrease in the factors responsible for MPTP opening such as oxidative stress and calcium overload. Pharmacological inhibition of the MPTP opening has been shown to reduce myocardial injury in ischaemia reperfusion heart models to protect the heart (Hausenloy *et al*. 2002). CsA, an immunosuppressant, has been shown to protect the myocardium from ischaemia reperfusion injury and oxidative stress via inhibition of the MPTP (Hausenloy *et al*. 2002, Hausenloy *et al*. 2003). CsA binds to Cyp-D and causes a conformational change in its morphology preventing it from binding to ANT thereby inhibiting MPTP opening (Tanveer *et al*. 1996).

We have shown in the previous chapter that the M_2 antagonist, AF-DX 116 exacerbates myocardial ischaemia-reperfusion injury via activating SAPK/JNK protein kinase and down-regulating the expression levels of miR-1 and miR-133b. The aim of this study was to investigate the effect of AF-DX 116 on MPTP activity using isolated cardiac myocytes subjected to oxidative stress. In addition, the effects of CsA will also be investigated in the isolated perfused rat heart calculating the infarct size, its effect on cell viability of cardiac myocytes undergoing hypoxia/re-oxygenation and also its effect on the MPTP. Finally, we will evaluate the effects of CsA in the absence and presence of AF-DX 116 on survival proteins such as Akt, ERK 1/2, and stress activated protein, SAPK/JNK.

5.2 Methods

5.2.1 Isolated perfused heart preparation

Sprague-Dawley rats were sacrificed (n=6) and the hearts were dissected as described in section 2.2. The hearts were mounted on the Langendorff system and perfused with KH buffer. LVDP, HR and CF were measured and recorded at regular intervals. The same procedure was followed as mentioned in sectioned 2.2 to calculate the percentage of infarct to risk ratio.

5.2.2 Langendorff protocol

The hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were administered at the onset and throughout reperfusion.

The hearts were randomly assigned to the following groups: a) hearts perfused with KH buffer alone with 30 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion

(control); b) hearts perfused with AF-DX 116 (1µM); c) hearts perfused with CsA (0.2µM); d) hearts perfused with co-administration of AF-DX 116 (1µM) and CsA (0.2µM). CsA has been previously shown to inhibit MPTP opening at a concentration of 0.2µM (Gharanei *et al*. 2014), hence we used the same concentration with AF-DX 116 (1µM) to investigate CsA induced protection against mAChR antagonist mediated injury.

5.2.3 MTT analysis of cell viability

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed (n=4) and the hearts were mounted on a Langendorff apparatus as described in section 2.3.1 to isolate cardiac myocytes. The procedure described in 2.3.2 was followed for the isolated cardiac myocytes to undergo hypoxia and re-oxygenation. The drugs were administered at the start of re-oxygenation. The cells were randomly assigned to the following treatment groups: a) Cells not treated with drug, and undergoing hypoxia for 2 hours and 2 hours of re-oxygenation (control); b) Cells treated with AF-DX 116 (1µM) administered at the onset of re-oxygenation following 2 hours of hypoxia; c) Cells treated with CsA (0.2µM) for 2 hours administered at the onset of re-oxygenation following 2 hours of hypoxia; d) Cells treated with the co-administration of AF-DX 116 (1µM) with CsA (0.2µM). The % cell viability of samples was calculated as mentioned in section 2.3.2.

5.2.4 Protocol to analyse MPTP opening

The protocol mentioned in section 2.4 was followed to measure the MPTP opening. Briefly, cardiac myocytes were isolated as mentioned in section 2.3.1. The viability of the cells was assessed by visualising the cells under a light microscope and the cell isolation yielding a viability of 65 % or more was used in the studies. The cardiac myocytes were plated onto glass cover slips coated with laminin to allow cell adhesion and incubated in microscopy buffer

containing TMRM (3μM). In this assay, TMRM localises in the negatively charged inner-membrane of the mitochondrion in a membrane potential-dependent manner where fluorescence is autoquenched (Perry *et al*. 2011). TMRM-loaded myocytes were then continuously illuminated at 543 nm which results in photo-sensitisation of TMRM generating ROS. Accumulation of ROS induces opening of the MPTP. The opening of the MPTP allows TMRM to leave the mitochondria and dequench in the cytosol causing depolarisation which is observed as an increase in fluorescence and reflects the opening of the MPTP (Hausenloy *et al.* 2004, Storey *et al.* 2013). An image showing a view of TMRM-loaded ventricular myocytes under a confocal microscope is shown in figure 5.1.

Sustained opening of the MPTP can lead to hypercontracture i.e. sustained shortening and stiffening of the cardiac myocytes which can lead to necrotic cell death (Piper *et al.* 2004). Reperfusion-induced hypercontracture can occur via two distinct mechanisms; calcium overload or reduced levels of cytosolic ATP (Abdallah e*t al.* 2010). The latter is a calcium-independent mechanism and is also known as rigor contracture. Rigor contracture occurs when cytosolic ATP concentration is reduced to a low (<100 μM) but non-zero level (Altschuld *et al.* 1985, Nichols and Lederer 1990). It is believed to be caused by activation of actin-myosin interactions along actin filaments at which rigor bonds are already formed at some of their various crossbridge sites (Piper *et al.* 2006). Studies have shown that strategies reducing the time to hypercontracture protect the cardiac myocytes from various myocardial insults including ischaemia reperfusion injury (Gharanei *et al.* 2012).

The cells were then incubated in the absence or presence of drugs for 10 minutes before being placed on the confocal microscope. Cells were randomly assigned to the following drug treatment groups: a) Cells not treated with drug (control); b) Cells treated with AF-DX 116

(1µM); c) Cells treated CsA (0.2µM); d) Cells treated with a combination of AF-DX 116 (1µM) with CsA (0.2µM).

The cells were viewed under a Zeiss 510 CLSM confocal microscope equipped with 20x objective lens (NA 1.3) and a heated stage. A 585-nm long pass filter allowed detection of TMRM. Laser stimulation via the 543-nm emission line of a HeNe laser was used to induce oxidative stress. Recording and analysis was facilitated by use of the Zeiss software package, LSM 2.8. The time of cells to undergo both, depolarisation and hypercontracture was recorded.

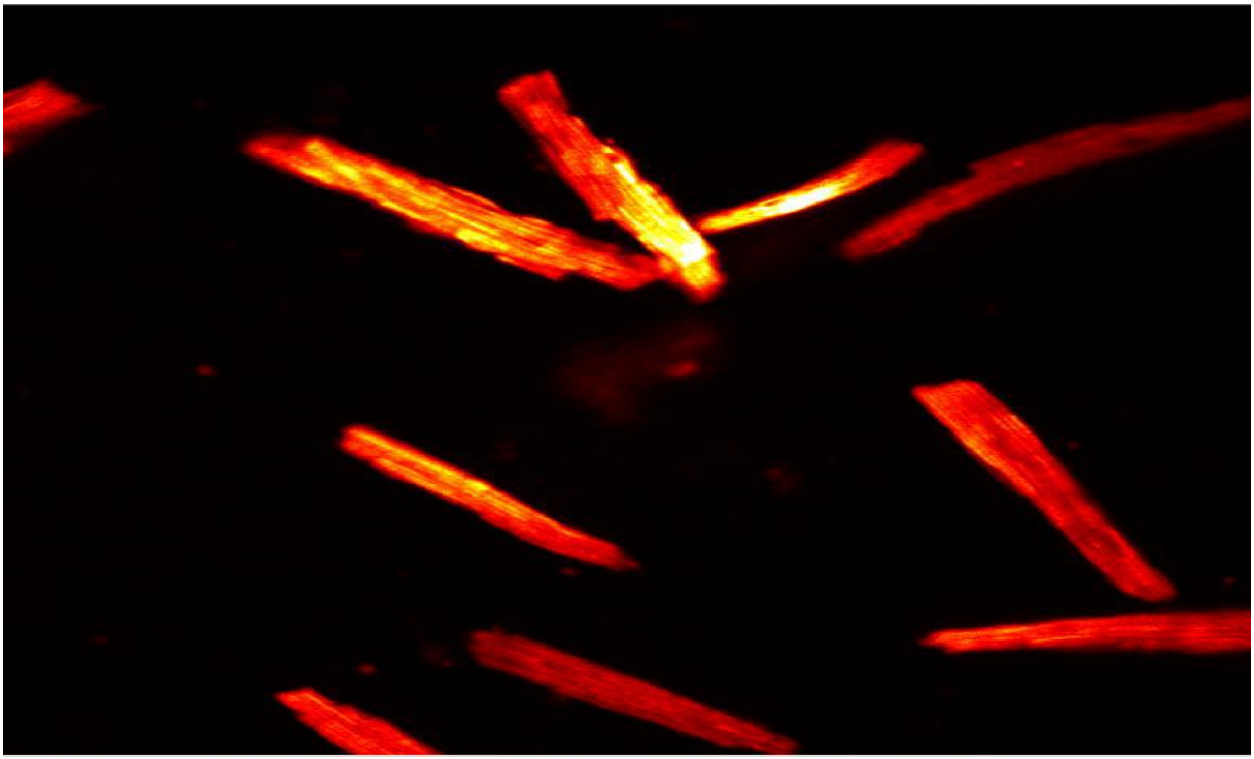

Figure 5.1: Image showing TMRM-loaded ventricular myocytes view under a confocal microscope at magnification x40

5.2.5 Western blot analysis of the isolated perfused heart tissue following drug treatment

Western blot analyses were carried out as mentioned in section 2.5. The samples were randomly assigned to the following experimental groups: a) hearts perfused with KH buffer alone with 35

minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with AF-DX 116 at a concentration range of 0.1µM-3µM; c) hearts perfused with ACh (0.1µM); d) hearts perfused with co-administration of AF-DX 116 (1µM) and ACh (0.1µM). After separation, the proteins were transferred onto the PVDF membrane and probed for the phosphorylated and the total form of Akt (Ser_{473}), ERK 1/2 (Thr_{202}/ Tyr_{204}) and SAPK/JNK (Thr_{183}/Tyr_{185}). The relative changes in the phosphorylated protein levels were calculated and corrected for differences in protein loading as established by probing for total form of Akt, ERK1/2 and SAPK/JNK into 20 minutes of reperfusion phase.

5.2.6 Statistical analysis

All values were expressed as mean ± SEM. Infarct size and cell viability were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Haemodynamics were assessed for statistical difference using two way ANOVA with Fishers post hoc tests using SPSS 12. The time taken to depolarisation and hypercontracture were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Differences were considered significant at $P \leq 0.05$.

5.3 Results

5.3.1 The effects of CsA ± AF-DX 116 on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

The effects of CsA in the absence and presence of AF-DX 116 on the infarct size of the hearts following ischaemia reperfusion injury were investigated. The results (figure 5.2) showed that hearts treated with CsA (0.2µM) had a significantly smaller infarct size as compared to the

untreated control [36.5 ± 1.99% (CsA, 0.2µM) vs. 47 ± 2.1% (control), $p<0.001$]. Also, as previously mentioned in section 4.3.1, AF-DX 116 (1µM) significantly increased infarct size as compared to the control [63.19 ± 1.13% (AF-DX 116, 1µM) vs. 47 ± 2.1 % (control), $p<0.001$]. Interestingly, the co-administration of CsA (0.2µM) with AF-DX 116 (1µM) abrogated the AF-DX 116 induced myocardial injury [51 ± 2.28% (AF-DX 116, 1µM and CsA, 0.2µM) vs. 63.19 ± 1.13% (AF-DX 116), $p<0.01$]. Although CsA was dissolved in ethanol but due to time constraint a vehicle control with ethanol was not performed. The data are shown in table 5.1.

Table 5.1: The effect of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on the infarct size to risk ratio as compared to the control and the relative SEM values (n=6).

Group	Control	AFDX (1µM)	CsA (0.2µM)	CsA (0.2µM) + AFDX (1µM)
Infarct size (%)	47	63.19	36.5	51
SEM	2.1	1.13	1.99	2.28

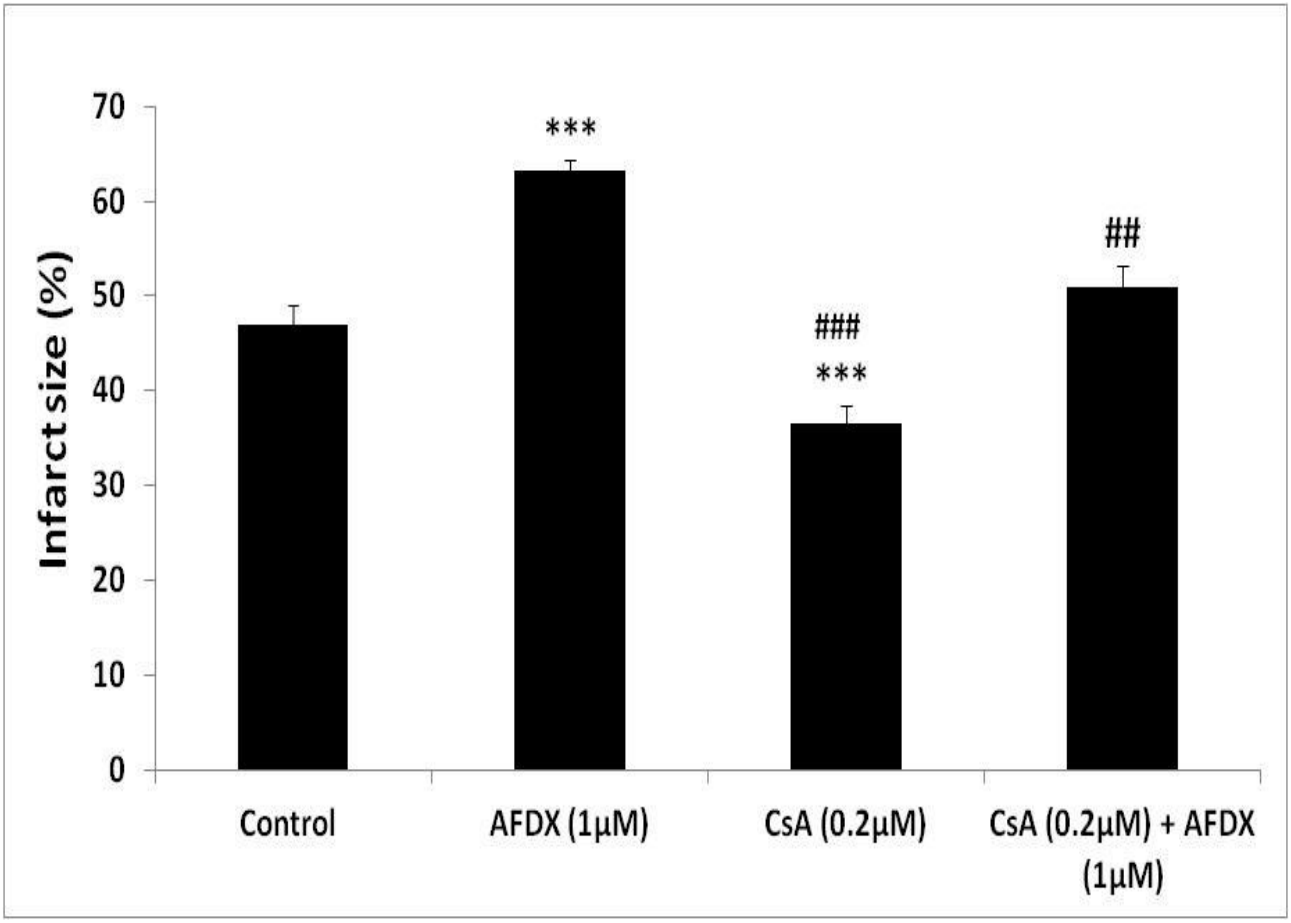

Figure 5.2: The effects of no drug treatment (control), AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. Control. ##p<0.01 and ###p<0.001 vs. AF-DX 116 (1µM).

5.3.2 The effects of CsA ± AFDX 116 on the haemodynamics of the heart

The haemodynamics including the LVDP, HR and CF of the hearts from the Langendorff model were recorded and measured. LVDP was calculated as the difference between the systolic pressure and the diastolic pressure and presented as a percentage of mean stabilisation. The effects of CsA (0.2µM) ± AF-DX 116 (1µM) treatment on the LVDP are shown in figure 5.3. The results showed that the AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the LVDP as compared to the untreated control.

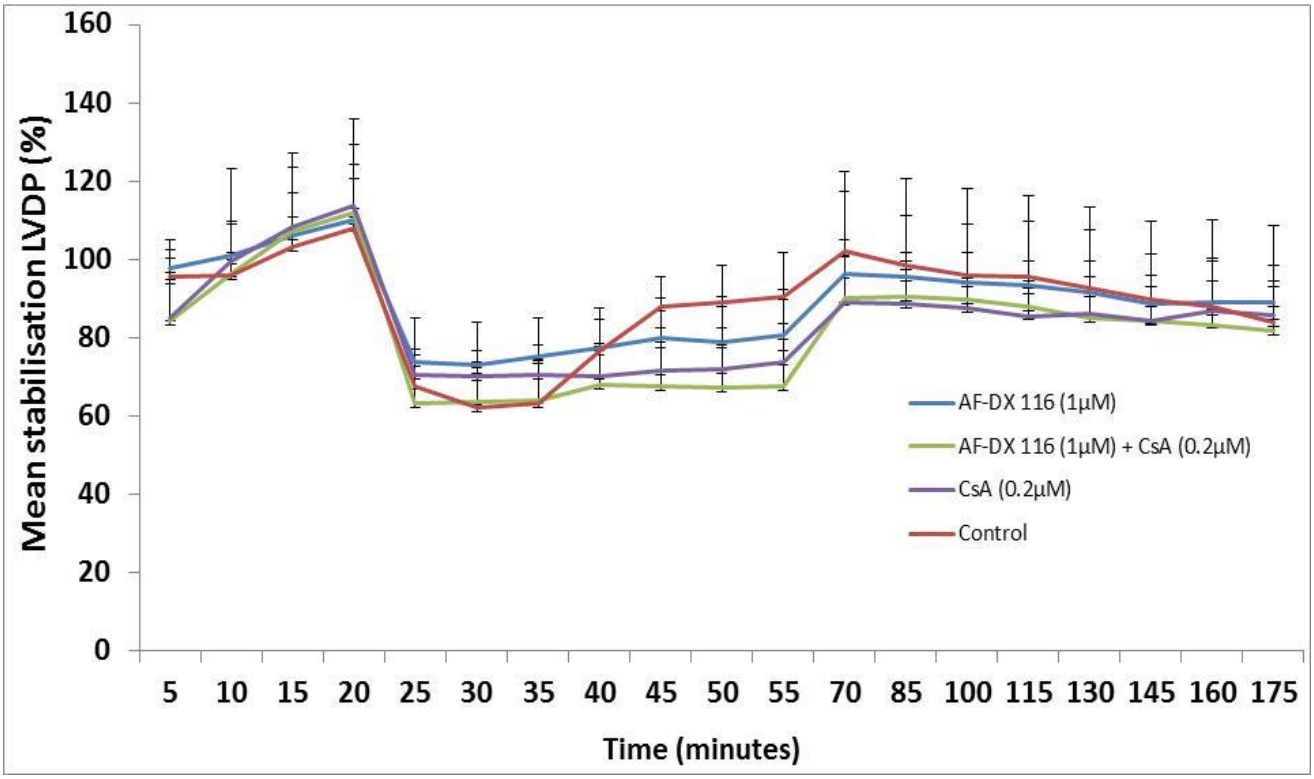

Figure 5.3: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on LVDP as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

The effects of AF-DX 116 (1µM) ± CsA (0.2µM) treatment on the heart rate are shown in figure 5.4. The results showed that the AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the heart rate as compared to the untreated control.

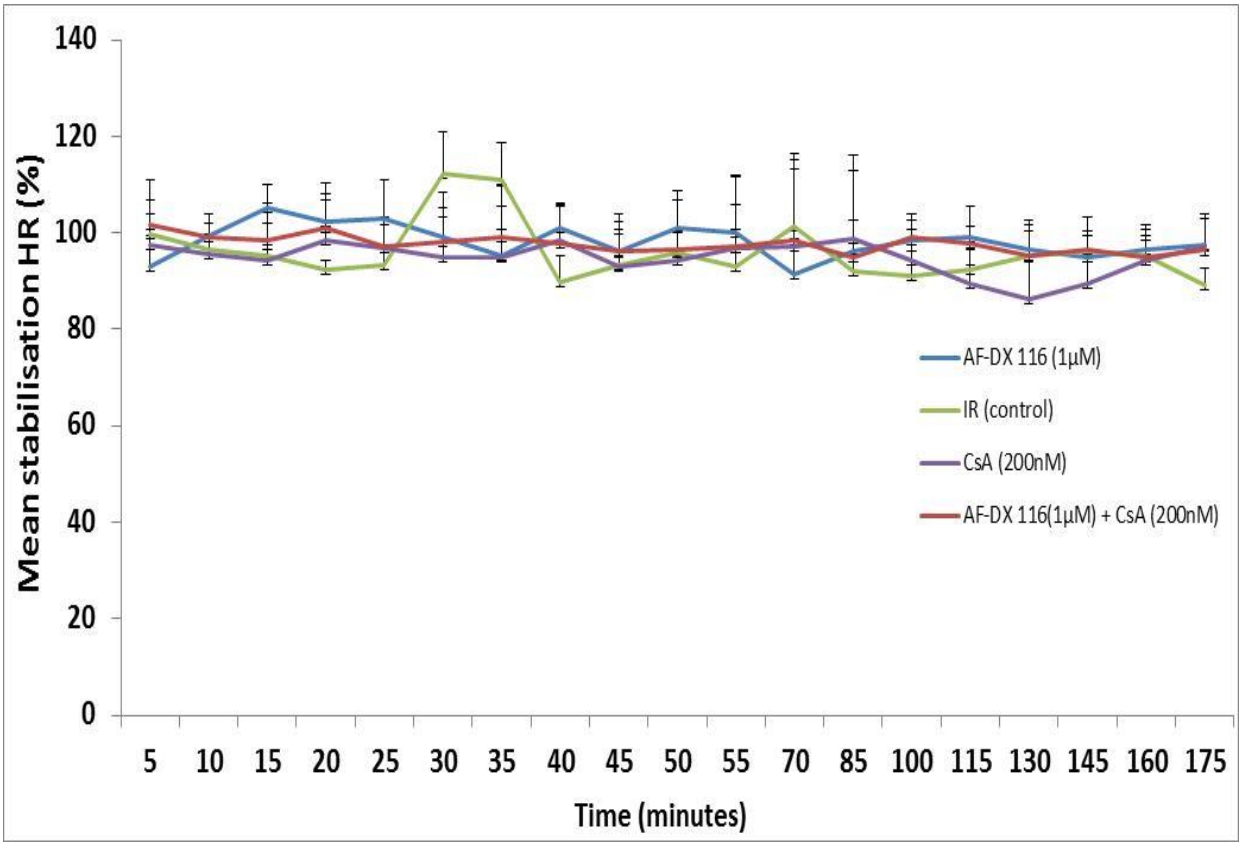

Figure 5.4: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on heart rate as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

CF was recorded by collecting the effluent for 1 minute at regular intervals; data presented are calculated, corrected for the heart weight and plotted as a percentage of mean stabilisation. The effects of AF-DX 116 (1µM) ± CsA (0.2µM) treatment on the CF are shown in figure 5.5. The results showed that the AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the CF as compared to the untreated control.

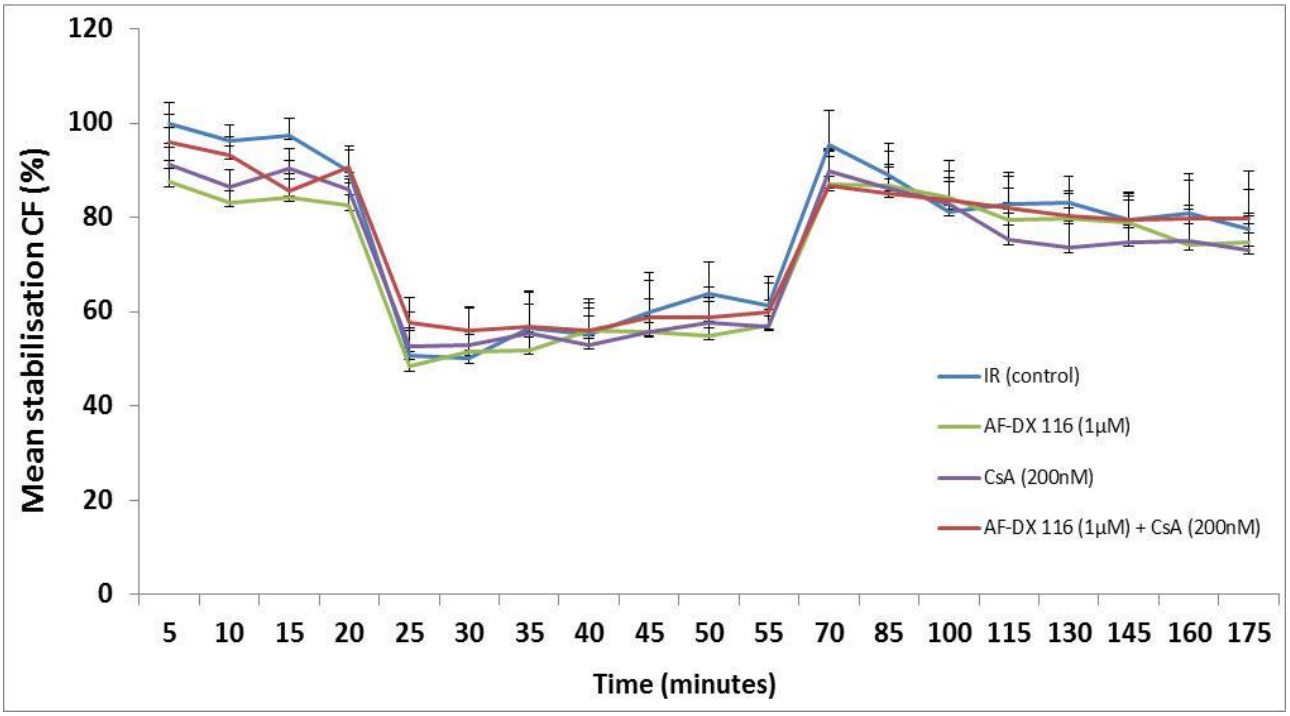

Figure 5.5: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on CF as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

5.3.3 The effects of CsA on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

Cardiac myocytes were isolated following the protocol mentioned in section 2.3.1 and were incubated with MTT in the dark for 2 hours to assess the cell viability. The reduction of MTT to formazan by mitochondrial dehydrogenase and corresponding colour change was indicative of the relative changes in myocytes survival and determined the cell viability. Figure 5.6 shows the effects of CsA (0.2µM) in the absence and presence of AF-DX 116 (1µM) on cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation. Drugs were added at the onset of re-oxygenation. As previously discussed in section 4.3.4, AF-DX 116 (1µM) significantly reduced the cell viability as compared to the control [70.5 ± 6.69% (AF-DX 116, 1µM) vs. 100 ± 0% (control), $p<0.05$]. Treatment with CsA (0.2µM) alone did not significantly change the

viability of cardiac myocytes. However, co-administration of CsA (0.2µM) with AF-DX 116 (1µM) abrogated the damage caused by AF-DX 116 (1µM) [83.35 ± 4% (AF-DX 116, 1µM and CsA, 0.2µM) vs. 70.5 ± 6.69% (AF-DX 116), $p<0.05$]. The data are shown in table 5.2.

Table 5.2: The effect of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	AFDX (1µM)	CsA (0.2µM)	CsA (0.2µM) + AFDX (1µM)
Cell Viability (%)	100	70.5	97.7	83.35
SEM	0	6.69	4.16	4

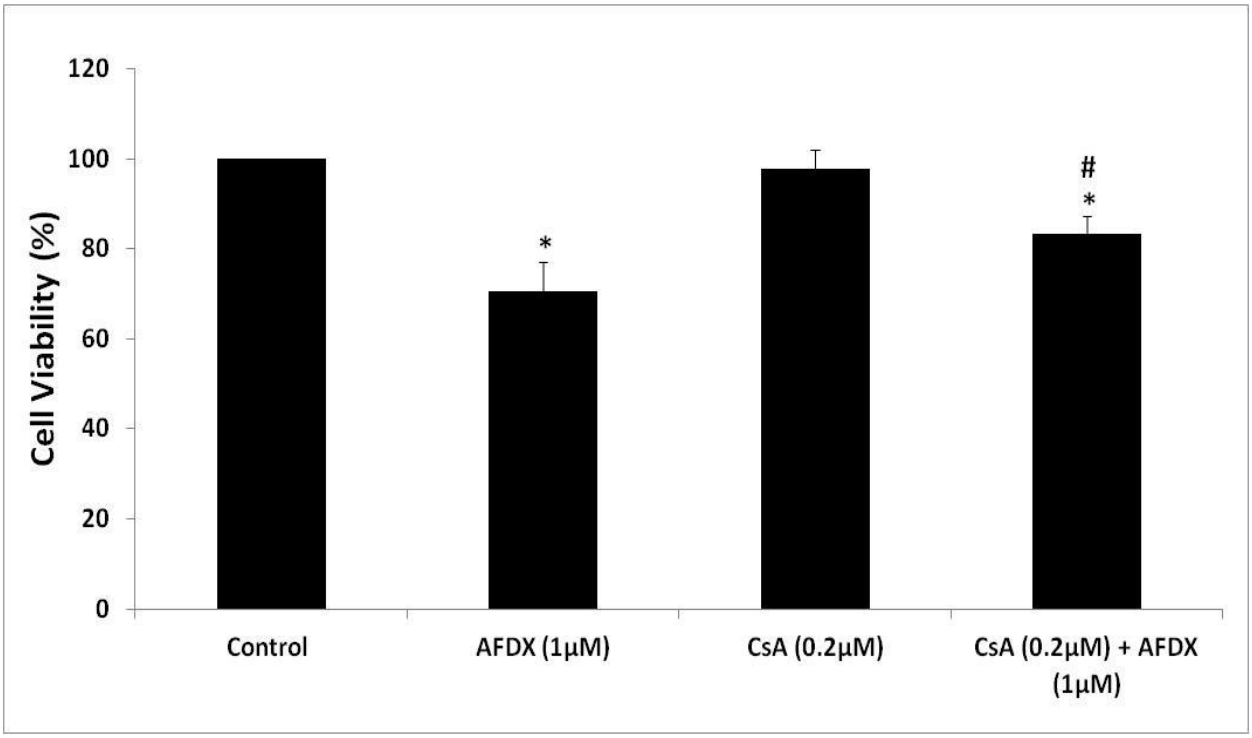

Figure 5.6: MTT analysis showing cell viability of cardiac myocytes in response to the treatment of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control and #p<0.05 vs. AF-DX 116 (1µM).

5.3.4 The effects of drug treatment on laser-induced oxidative stress in cardiac myocytes

Laser induced oxidative stress initiates mitochondrial depolarisation which indicates MPTP opening. Laser stimulation initiates photodecomposition of TMRM thus generating mitochondrial reactive oxygen species, leading to disruption of the mitochondrial membrane. Depolarisation begins as a wave of increased cytosolic TMRM at one end of the cell and propagates throughout the cell (Hausenloy *et al*. 2003). Continued oxidative stress leads to hypercontracture of the cell which signifies ATP depletion.

The effects of drug treatment on laser-induced oxidative stress in cardiac myocytes were investigated. The results (figure 5.7) showed that AF-DX 116 (1µM) significantly reduced the depolarisation time as compared to the control [191.8 ± 12 sec (AF-DX 116, 1µM) vs. 250.5 ± 12.62 sec (control), $p<0.001$]. Our results also showed that CsA (0.2µM) treatment alone significantly increased the depolarisation time as compared to the control [317.5 ±13.27 sec (CsA, 0.2µM) vs. 250.5 ± 12.62 sec (control), $p<0.001$]. Co-administration of CsA (0.2µM) with AF-DX 116 (1µM) significantly increased the time to depolarisation as compared to AF-DX 116 (1µM) alone [255.5 ± 16.07 sec (AF-DX 116, 1µM and CsA, 0.2µM) vs. 191.8 ± 12.04 sec (AF-DX 116), $p<0.01$]. The data are shown in table 5.3.

Table 5.3: The effect of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on depolarisation time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6).

Group	Control	AFDX (1µM)	CsA (0.2µM)	AFDX (1µM) + CsA (0.2µM)
Mean depolarisation time (sec)	250.5	191.8	317.5	255.5
SEM	12.62	12.04	13.27	16.07

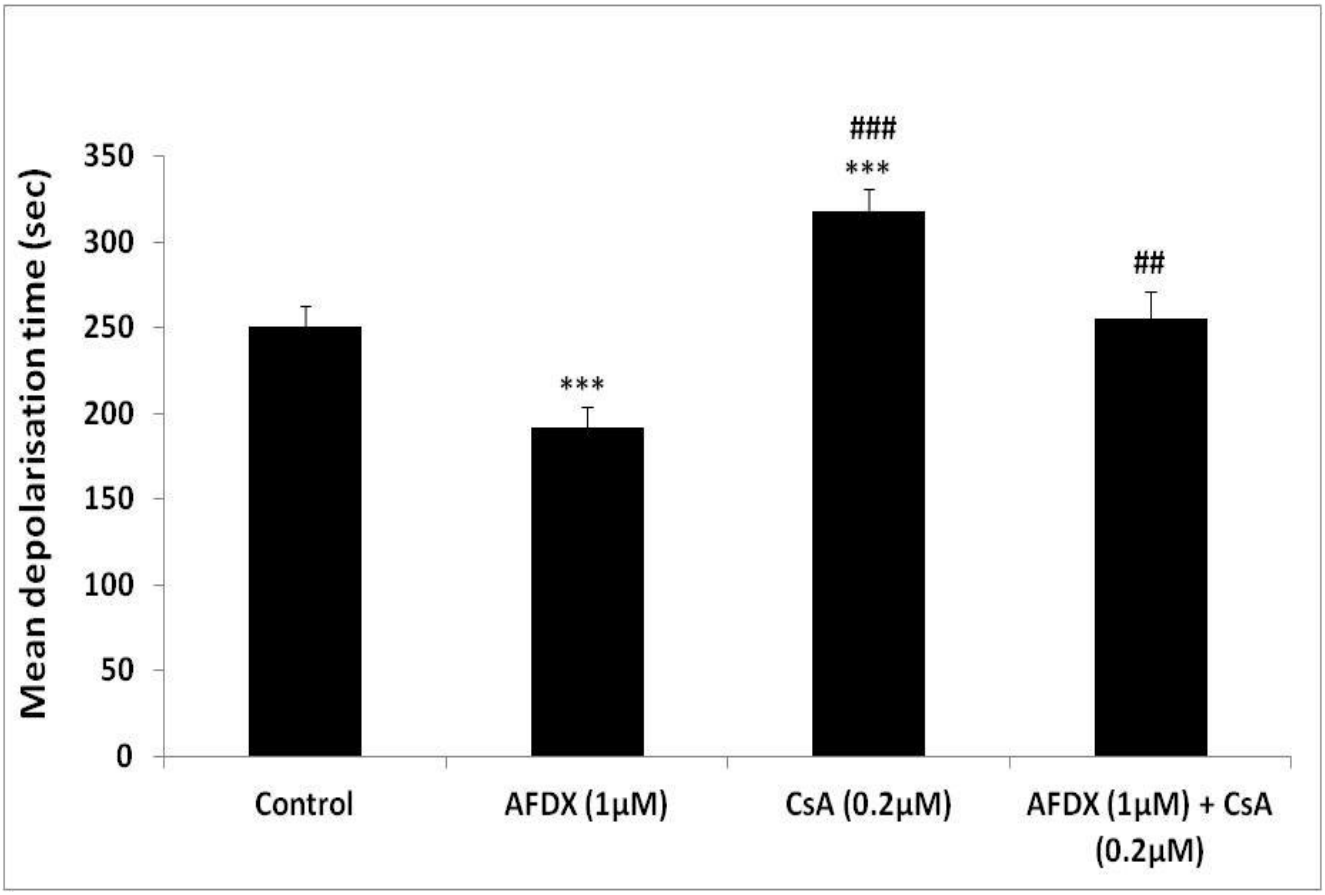

Figure 5.7: The effects of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on depolarisation time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. control, ##p<0.01 and ### = p<0.001 vs. AF-DX 116 (1µM).

AF-DX 116 (1µM) treatment of adult rat cardiac myocytes also significantly reduced the hypercontracture time as compared to the control (figure 5.8). Furthermore, CsA (0.2µM) treatment alone significantly increased the hypercontracture time as compared to the control (Table 5.4). Co-administration of CsA (0.2µM) with AF-DX 116 (1µM) significantly increased the time to hypercontracture as compared to AF-DX 116 (1µM) alone. The data are shown in table 5.4.

Table 5.4: The effect of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on hypercontracture time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6).

Group	Control	AFDX (1µM)	CsA (0.2µM)	AFDX (1µM) + CsA (0.2µM)
Mean hypercontracture time (sec)	741.67	553.83	873.33	724
SEM	21.48	25.15	22.94	33.94

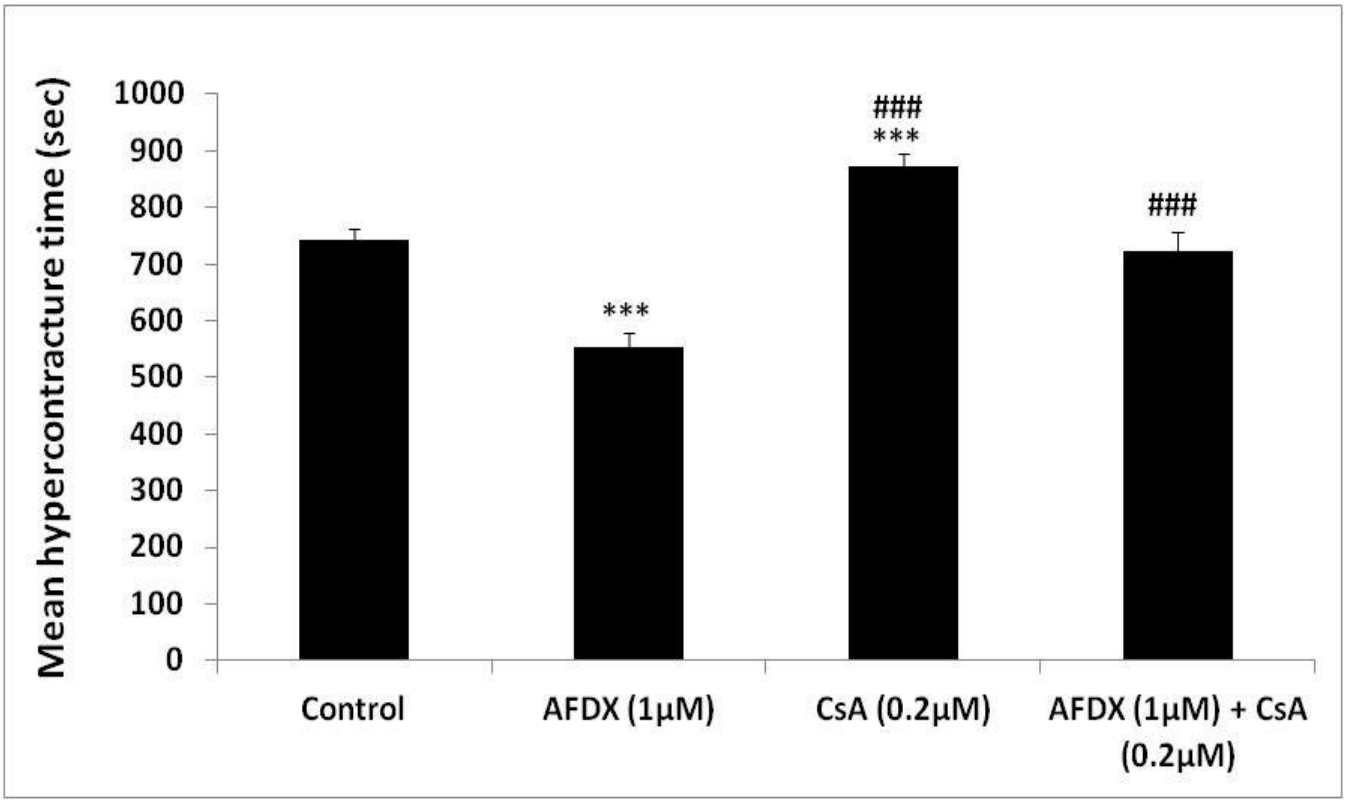

Figure 5.8: The effects of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on hypercontracture time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. control, ### = p<0.001 vs. AF-DX 116 (1µM).

5.3.5 The effects of CsA ± AF-DX 116 on the levels of signalling proteins as assessed by western blot analysis

To understand the molecular signalling mechanism via which CsA (0.2µM) was shown to reduce myocardial ischaemia reperfusion injury (section 5.3.1) we performed western blot analysis. The effects of CsA (0.2µM), in the absence and presence of AF-DX 116 (1µM), on the levels of

phosphorylated Akt, ERK and SAPK/JNK at 20 minutes into the reperfusion phase were investigated.

The results (figure 5.9) showed that AF-DX 116 (1µM) did not lead to any significant change in the levels of phosphorylated Akt as compared to the control. However, administration of CsA (0.2µM) at 20 minutes into reperfusion caused a significant increase in p-Akt levels as compared to the control [150.9 ± 17.5 % (CsA, 0.2µM) vs. 100 ± 0% (control), $p<0.01$]. Furthermore, the co-administration of CsA (0.2µM) with AF-DX 116 (1µM) led to significantly reduced p-Akt levels as compared to the (increased) levels observed with CsA (0.2µM) treatment alone [129.7 ± 7.2 % (CsA, 0.2µM + AF-DX 116, 1µM) vs. 150.9 ± 17.5 % (CsA, 0.2µM) $p<0.01$].

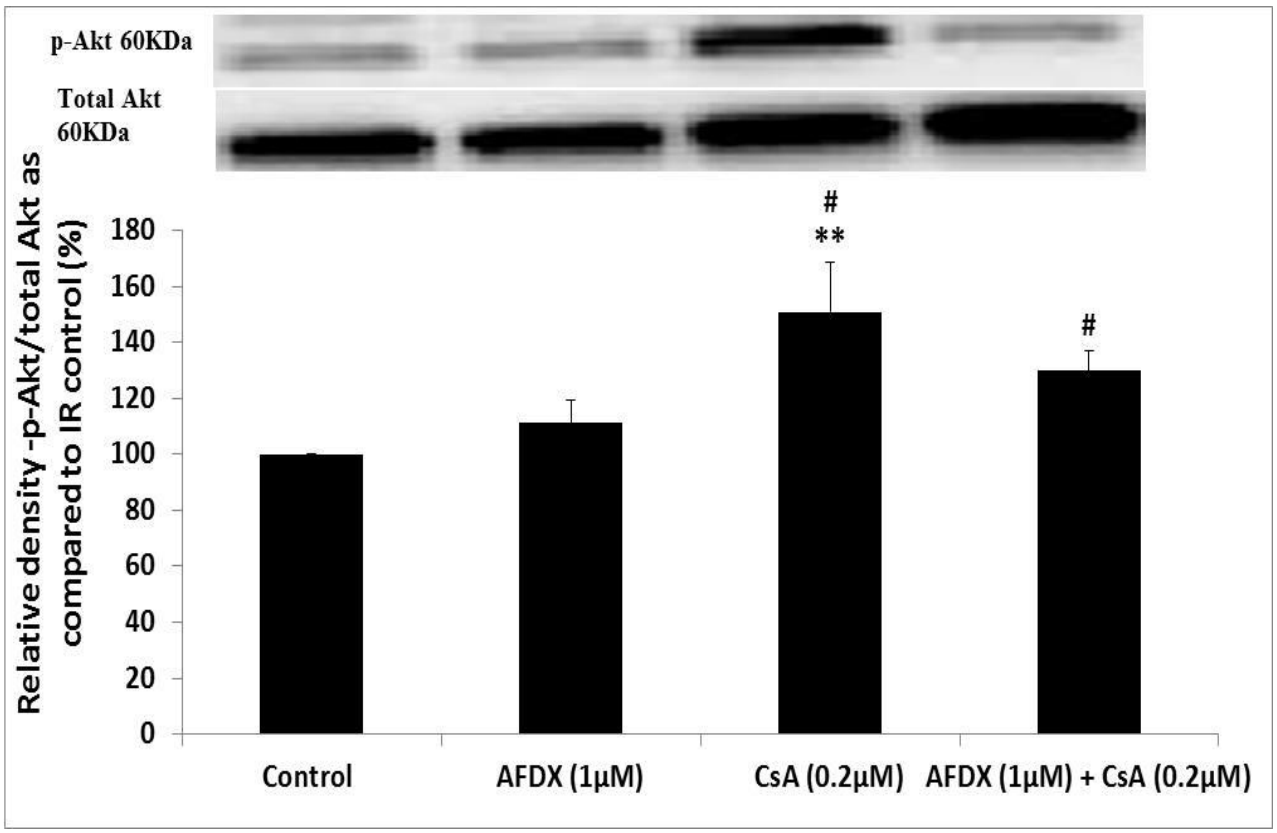

Figure 5.9: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 vs. Control, #p<0.05 vs. CsA (0.2µM).

In addition, the results (figure 5.10) also showed that AF-DX 116 (1µM) did not lead to any significant change in the levels of phosphorylated ERK 1/2 as compared to the control. However, administration of CsA (0.2µM) at 20 minutes into reperfusion caused a significant increase in p-ERK 1/2 levels as compared to the control [160.5 ± 10.7 % (CsA, 0.2µM) vs. 100 ± 0 % (control), $p<0.01$].

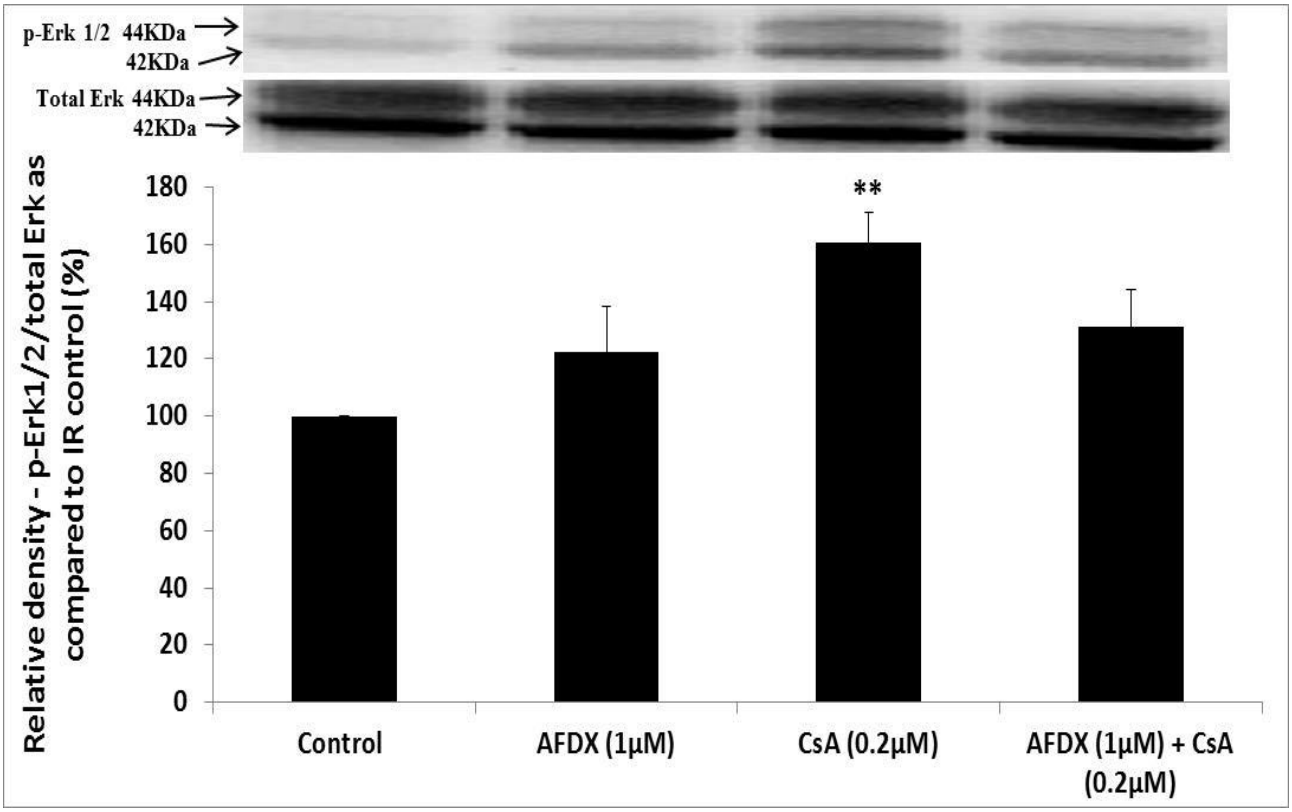

Figure 5.10: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.01 vs. Control.

As also observed in section 4.3.6, AF-DX 116 (1µM) led to significantly increased p-SAPK/JNK levels as compared to the control [171.9 ± 7% (AF-DX 116, 1µM) vs. 100 ± 0% (control) $p<0.05$]. In addition, results (figure 5.11) showed that CsA (0.2µM) led to significantly reduced p-SAPK/JNK levels as compared to the control [66.6 ± 6.1 % (CsA, 0.2µM) vs. 100 ± 0% (control) $p<0.01$]. Furthermore, the co-administration of AF-DX 116 (1µM) with CsA (0.2µM)

led to significantly reduced p-SAPK/JNK levels as compared to the (increased) levels observed by AF-DX 116 (1µM) alone [89.76 ± 4.09 % (CsA, 0.2µM + AF-DX 116, 1µM) vs. 171.9 ± 7% (AF-DX 116, 1µM) $p<0.01$]. The data are shown in table 5.5.

Table 5.5: The effect of AF-DX 116 (1µM), CsA (0.2µM) and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on expression levels of p-SAPK/JNK. Results are expressed as mean ± SEM (n=3).

Group	Control	AFDX (1µM)	CsA (200nM)	AFDX (1µM) + CsA (200nM)
p-SAPK/JNK/Total SAPK/JNK (%)	100	171.9	66.63	89.76
SEM	0	7	6.15	4.09

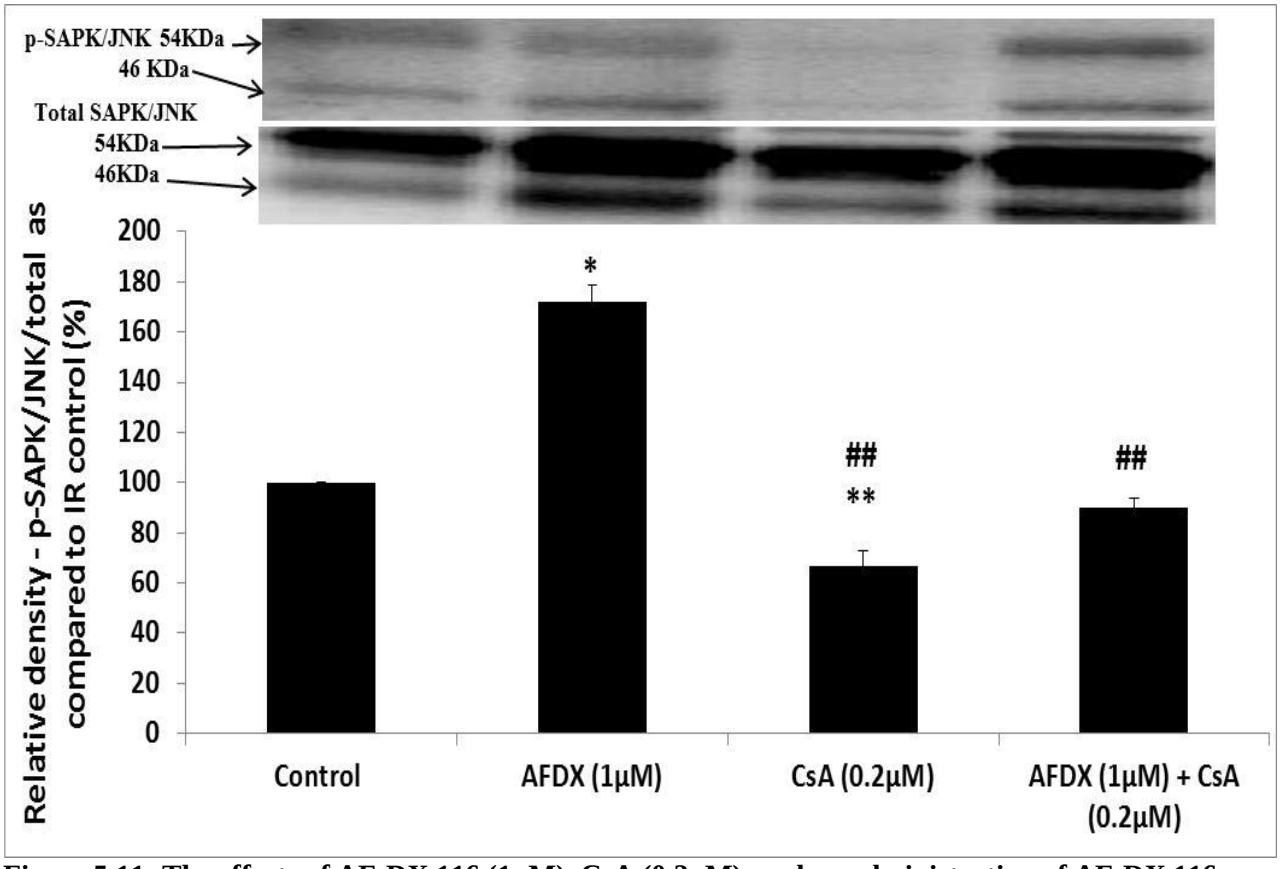

Figure 5.11: The effects of AF-DX 116 (1µM), CsA (0.2µM), and co-administration of AF-DX 116 (1µM) with CsA (0.2µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). **p<0.01 vs. Control, ##p<0.01 vs.AFDX (1µM).

5.4 Discussion

We have shown in the previous chapter that inhibiting the M_2 mAChR by AF-DX 116 exacerbates myocardial ischaemia- reperfusion injury via activating SAPK/JNK and down-regulating the expression levels of miR-1 and miR-133b. Mitochondrial dysfunction has been shown to be an underlying cause of ischaemia-reperfusion injury (Crompton *et al*. 1999). It is involved in mediating lethal permeability changes to initiate cell death and has also been shown to be involved in drug induced myocardial injury by our lab (Gharanei *et al*. 2013). We therefore investigated the effect of AF-DX 116 on MPTP in isolated rat cardiac myocytes.

The current study indicates that the M_2 muscarinic receptor antagonist AF-DX 116 significantly exacerbates myocardial injury in *ex vivo* conditions of oxidative stress by premature opening of the MPTP. AF-DX 116 led to significantly reduced depolarisation and hypercontracture time of isolated cardiac myocytes resulting in cell death. As previously discussed in section 4.4, and also shown in this study (5.3.1), AF-DX 116 exacerbates myocardial ischaemia reperfusion injury. The current study indicates a novel mechanism for AF-DX 116 induced cardiotoxicity in stress conditions. We postulate that these observations suggest a central role of MPTP in AF-DX 116 induced toxicity in our models of ischaemia reperfusion injury.

Opening of the MPTP is known to be involved in various pathological conditions including ischaemia reperfusion injury (Yellon and Hausenloy 2007). MPTP remain closed during ischaemia and has been shown to open during early minutes of reperfusion when conditions that increase the probability of its opening prevail such as high mitochondrial calcium, build-up of ROS and inorganic phosphate load (Griffiths and Halestrap 1995, Di Lisa *et al*. 2001). These conditions disrupt the osmotic barrier between the mitochondria and the cytosol and upon MPTP opening, allow free passage of molecules smaller than 1.5KDa in size (Halestrap and Pasdois

2009, Yellon and Hausenloy 2007). This initiates a colloidal osmotic pressure on the mitochondrial membrane which leads to cell swelling and eventual rupture of the outer mitochondrial membrane releasing pro-apoptotic factors such as cytochrome c in the inter-membrane space (Halestrap *et al*. 2004). MPTP opening also allows permeability to protons, which results in uncoupling of oxidative phosphorylation and consequently, ATP depletion. This in turn can activate degradative enzymes such as phospholipases and proteases and also disrupt ionic and metabolic homeostasis (Halestrap *et al*. 2002). These effects can lead to irreversible cell damage and eventually resulting in necrotic death.

We have also demonstrated that the MPTP blocker CsA, protected the heart from the damage caused by ischaemia reperfusion injury alone in the Langendorff studies. CsA has been previously shown to reduce infarction in ischaemic reperfusion heart models thereby protecting the myocardium (Crompton *et* al. 1988, Griffiths and Halestrap 1993, Shanmuganathan *et* al. 2005). Interestingly, our data demonstrates that the co-administration of CsA with AF-DX116 abrogated the injury inflicted by AF-DX 116 during ischaemia reperfusion injury. The protective effects of CsA were further confirmed by the findings of the laser induced oxidative stress model. The results showed that CsA alone delayed the depolarisation and hypercontracture time of cardiac myocytes and also reversed the injury induced by AF-DX 116.

CsA binds with high affinity to Cyp-D, initiating a conformational change in Cyp-D morphology (Tanveer *et al*. 1996), thereby inhibiting the binding of Cyp-D to ANT and preventing subsequent MPTP opening (Basso *et al*. 2005). CsA has also been shown to significantly reduce infarct size in a coronary occlusion model of reperfusion injury (Hausenloy

et al. 2002).Taken together, these data strongly support that CsA is an effective means of inhibiting reperfusion injury by direct inhibition of the MPTP.

The current study also demonstrates that CsA activates pro-survival kinases Akt and ERK1/2 and inhibits stress activated protein, SAPK/JNK, to protect myocardium against ischaemia reperfusion injury. Phosphorylation of Akt and ERK1/2 is known to be integral to the reperfusion injury salvage kinase pathway that converges on the MPTP (Davidson *et al*. 2006, Hausenloy and Yellon 2007). Ischaemic and pharmacological pre-conditioning is found to protect the heart by phosphorylation of Akt and ERK 1/2 (Hausenloy *et al*. 2005, Hu *et al.* 2008). Studies have also shown that CsA is involved in the activation of pro-survival proteins such as Akt and ERK. Han *et al*. (2010) showed that CsA enhances keratinocyte survival from removal of UVB radiation via Akt activation in HaCaT mice cells. Furthermore, Yang *et al*. (2003) studied the effect of CsA preconditioning in ischaemic rat kidneys and showed that CsA significantly increased ERK expression and decreased stress activated protein, JNK. CsA has also been shown to significantly inhibit activation of stress protein kinase, SAPK1 in human peripheral blood mononuclear cells (Kreideweiss *et al*. 1999).

Taken together, this study provides the first evidence that AF-DX 116 induced cardiotoxicity is due to the opening of the MPTP in the setting of ischaemia reperfusion injury. The collective findings of the previous chapter and present study have provided a detailed understanding of the molecular mechanism involved in AF-DX 116 induced injury to the myocardium. We have also shown that CsA has not only protected the myocardium from the injury but has also reversed the damage mediated by AF-DX 116. Non-selective mAChR antagonists, such as ipratropium bromide are routinely used to treat respiratory conditions such as COPD; however, such

antagonists have been shown to exhibit severe cardiovascular risks. As the association between COPD and cardiovascular disease has been discussed in detail in section 1.1, these findings about CsA may demonstrate a potential adjunctive therapy route for such patients who may have underlying ischaemic heart disease. Such claims would of course warrant further investigations.

Chapter Six: The M_3 muscarinic acetylcholine receptor antagonist, DAU 5884 exacerbates myocardial injury via activation of SAPK/JNK pathway

6.1 Introduction

We have previously shown (Chapter 4 and 5) that the inhibition of the M_2 mAChR by AF-DX 116 reveals cardiotoxic effects on the myocardium in the setting of ischaemia reperfusion injury. We also provided a detailed analysis of the signalling pathway proteins involved in the exacerbation of such an injury. As ipratropium bromide is a non-selective M_1-M_3 mAChR antagonist, it is important to study the involvement of each M_1-M_3 mAChR specifically. We have previously discussed the effects of M_1 and M_2 mAChRs in the setting of ischaemia reperfusion injury but the role of the M_3 mAChR in such an injury is not known.

The M_3 mAChR plays an important role in airway function by promoting increased tension and thereby airway narrowing in response to ACh (Roffel *et al*. 1990). Furthermore, M_3 mAChR have also been shown to be involved in the regulation of mucous secretion in submucosal glands (Rogers 2001) and in chemotactic mediator release in alveolar macrophages (Sato *et al*. 1998). The activation of the M_3 receptors is mainly involved in smooth muscle contraction of the airways which leads to bronchoconstriction (Eglen *et al*. 1996), and is therefore an important therapeutic target to treat pulmonary obstructions such as COPD.

Despite the M_2 subtype being the major population of mAChRs in the heart, there is evidence that the M_3 subtype is also present. The M_3 mAChR has been shown to be present in dog atrium (Shi *et al*. 1999; Wang *et al*. 1999) and also in adult rat cardiomyocytes (Ponicke *et al*. 2003). Molecular evidence suggests that M_3 mAChRs are present in human myocytes. Hellgren *et al*.

(2000) and Oberhauser *et al*. (2001) independently showed that despite the abundant presence of M_2 receptors, there was also an evidence of mRNA for the M_3 receptors in the left and right atria, and right ventricles of human hearts. In addition, Wang *et al*. (2001) also revealed the expression of the M_3 gene and localisation of the M_3 receptor protein in the cytoplasmic membrane of the human atria and ventricles.

The M_3 mAChRs play an important role in the regulation and maintenance of cardiac function (Liu *et al*. 2001, Yue *et al*. 2006). Studies have shown that the cardiac M_3 receptors are involved in the regulation of heart rate and cardiac repolarisation (Yang *et al*. 2005), modulation of inotropic effects (Nishimaru *et al*. 2000), regulation of cell-to-cell communication (Yue *et al*. 2006) and in generation and maintenance of atrial fibrillation (Dobrev *et al*. 2002).

To understand the involvement of individual M_3 mAChR in ischaemia reperfusion injury, we investigated the effects of M_3 mAChR antagonist DAU 5884 hydrochloride in the absence and presence of mAChR agonist ACh in a whole heart Langendorff model of ischaemia-reperfusion and also the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation. In addition, the effects of DAU 5884 on cell signalling protein kinases such as p-Akt, p-ERK 1/2, and p-SAPK/JNK were also investigated.

6.2 Methods

6.2.1 Isolated perfused heart preparation

Sprague-Dawley rats were sacrificed and the hearts were dissected as described in section 2.2. The hearts were mounted on the Langendorff system and perfused with KH buffer. LVDP, HR and CF were measured and recorded at regular intervals. The same procedure was followed as mentioned in sectioned 2.2 to calculate the percentage of infarct to risk ratio.

6.2.2 Langendorff protocol

The hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were administered at the onset of, and throughout reperfusion.

The hearts were randomly assigned to the following groups: a) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with DAU 5884 hydrochloride at a concentration range of 0.001µM-3µM; c) hearts perfused with ACh (0.1µM); d) hearts perfused with co-administration of DAU 5884 (1µM) and ACh (0.1µM). DAU 5884 is a selective M_3 mAChR antagonist (Gosens *et al*. 2003).

The exact administration dosage of ipratropium bromide to the patient varies; but is usually administered at a range of 40µg-500µg (Boehringer Ingelheim 1987). As ipratropium bromide is a non-selective M_1-M_3 mAChR antagonist with bioavailability of only 7% in humans, we aimed to use a wide concentration range of 0.001µM-3µM for DAU 5884 in our study to block the M_3 mAChR at clinically relevant concentrations.

6.2.3 MTT analysis of cell viability

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed (n=4) and the hearts were mounted on a Langendorff apparatus as mentioned in section 2.3.1 to isolate cardiac myocytes. The procedure mentioned in 2.3.2 was followed for the isolated cardiac myocytes to undergo hypoxia and re-oxygenation. The drugs were administered at the start of re-oxygenation. The cells were randomly assigned to the following treatment groups: a) Cells not treated with drug, and undergoing hypoxia for 2 hours and 2 hours of re-oxygenation (control); b) Cells treated

with DAU 5884 at a concentration range of 0.003µM-3µM administered at the onset of re-oxygenation following 2 hours of hypoxia; c) Cells treated with ACh (0.1µM) for 2 hours at a concentration range of 0.01µM-1µM administered at the onset of re-oxygenation following 2 hours of hypoxia; d) Cells treated with the co-administration of DAU 5884 (1µM) with ACh (0.1µM). The % cell viability of samples was calculated as described in section 2.3.2.

6.2.4 Western blot analysis of the isolated perfused heart tissue following drug treatment

Western blot analyses were carried out as mentioned in section 2.5. The samples were randomly assigned in the following experimental groups: a) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with DAU 5884 (1µM); c) hearts perfused with ACh (0.1µM); d) hearts perfused with co-administration of DAU 5884 (1µM) and ACh (0.1µM). After separation, the proteins were transferred onto the PVDF membrane and probed for the phosphorylated and the total form of Akt (Ser_{473}), ERK 1/2 (Thr_{202}/ Tyr_{204}) and SAPK/JNK (Thr_{183}/Tyr_{185}). The relative changes in the phosphorylated protein levels were calculated and corrected for differences in protein loading as established by probing for total form of Akt, ERK 1/2 and SAPK/JNK into 20 minutes of reperfusion phase.

6.2.5 Statistical analysis

All values were expressed as mean ± SEM. Infarct size and cell viability were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Haemodynamics were assessed for statistical difference using two way ANOVA with Fishers post hoc tests using SPSS 12. Differences were considered significant at $P \leq 0.05$.

6.3 Results

6.3.1 The effects of DAU 5884 on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

Whole hearts undergoing ischaemia and reperfusion were used to calculate the infarct size to risk ratio from the Langendorff model. The hearts were treated with a M_3 mAChR antagonist, DAU 5884 (0.001µM-3µM) which was added at the onset of reperfusion. The infarct size was calculated as described in section 2.2. The results as shown in figure 6.1 showed that DAU 5884 (0.1µM-3µM) led to increased infract size in a dose dependent manner as compared to the untreated control [57.5 ± 1.28 % (DAU 5884, 0.1µM) vs. 47.21 ± 2.1 % (control) $p<0.01$, 59.1 ± 1.78 % (DAU 5884, 0.3µM) vs. 47.21 ± 2.1 % (control) $p<0.001$, 67.8 ± 1.61 % (DAU 5884, 1µM) vs. 47.21 ± 2.1 % (control) $p<0.001$, 61.5 ± 1.59 % (DAU 5884, 3µM) vs. 47.21 ± 2.1 % (control) $p<0.01$]. The data are shown in table 6.1.

Table 6.1: The effect of DAU 5884 (0.001µM-3µM) on the infarct size to risk ratio as compared to the IR control and the relative SEM values (n=6).

Group	Control	0.001µM	0.003µM	0.01µM	0.03µM	0.1µM	0.3µM	1µM	3µM
Infarct size (%)	47	49.7	50.83	51.67	54	57.5	59.16	67.83	61.5
SEM	2.1	1.28	1.05	1.05	0.98	1.28	1.78	1.61	1.59

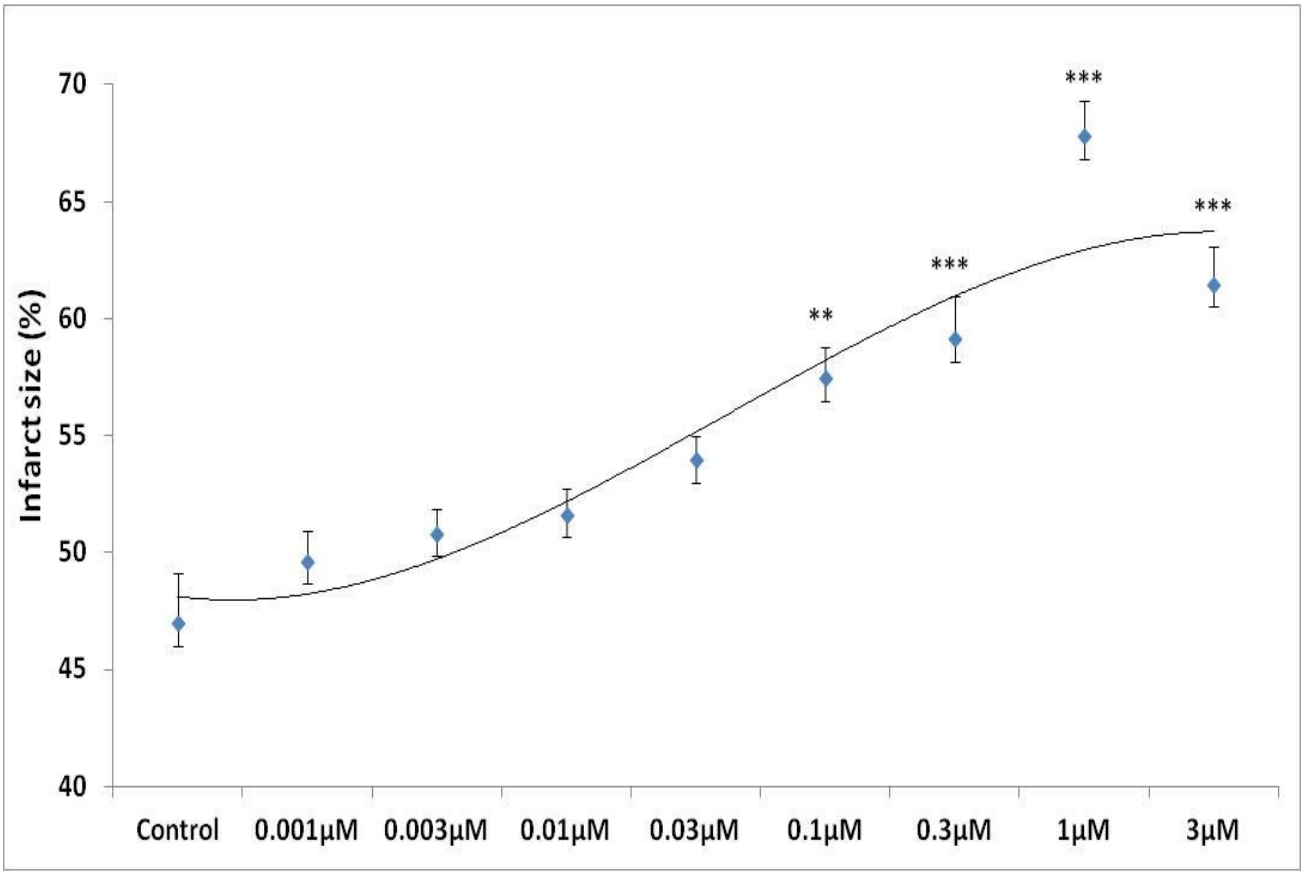

Figure 6.1: The effects of no drug treatment (IR control) and DAU 5884 (0.001µM-3µM) on the infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). *p<0.05, and ***p<0.001 vs. Control.

6.3.2 The effects of DAU 5884 ± ACh on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

ACh is a natural mAChR agonist that has been shown to provide protection against various cellular insults including myocardial ischaemia reperfusion injury (Yang *et al*. 2005). We therefore investigated the effects of ACh (0.1µM) and its co-administration with the M_3 antagonist, DAU 5884 hydrochloride (1µM) on hearts undergoing ischaemia reperfuion injury. The results (figure 6.2) showed that ACh (0.1µM) significantly reduced the infarct size in comparison to the control [39 ± 2.11 % (ACh, 0.1µM) vs. 47 ± 2.09 % (control), $p<0.01$]. Interestingly, the observed increase in infarct size due to DAU 5884 treatment (1µM) was

significantly attenuated when co-administered with ACh (0.1µM) [53 ± 1.72 % (ACh, 0.1µM) vs. 67.8 ± 1.61 % (DAU 5884, 1µM), p<0.01].

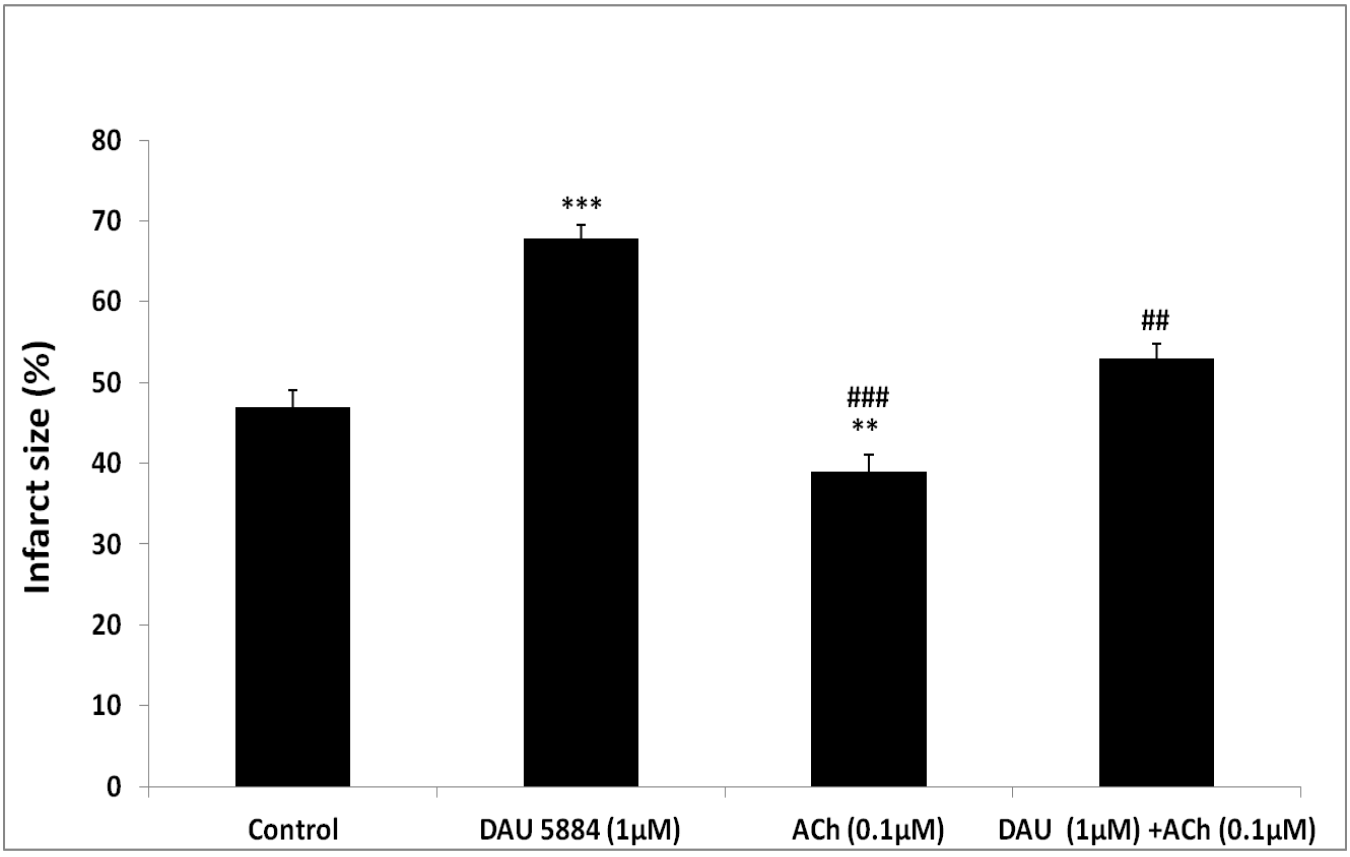

Figure 6.2: The effects of no drug treatment (control), DAU 5884 (1µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) and ACh (0.1µM) on infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). **p<0.01 and ***p<0.001 vs. Control, ##P<0.01 and ###p<0.001 vs. AF-DX 116 (1µM).

6.3.3 The effects of DAU 5884 ± ACh on the haemodynamics of the heart

The haemodynamics including the LVDP, HR and CF of the hearts from the Langendorff model were recorded and measured. LVDP was calculated as the difference between the systolic pressure and the diastolic pressure and presented as a percentage of mean stabilisation. The effects of DAU 5884 (1µM) ± ACh (0.1µM) treatment on the LVDP are shown in figure 6.3. The results showed that the DAU 5884 (1µM), ACh (0.1µM) and co-administration of DAU

5884 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the LVDP as compared to the untreated control.

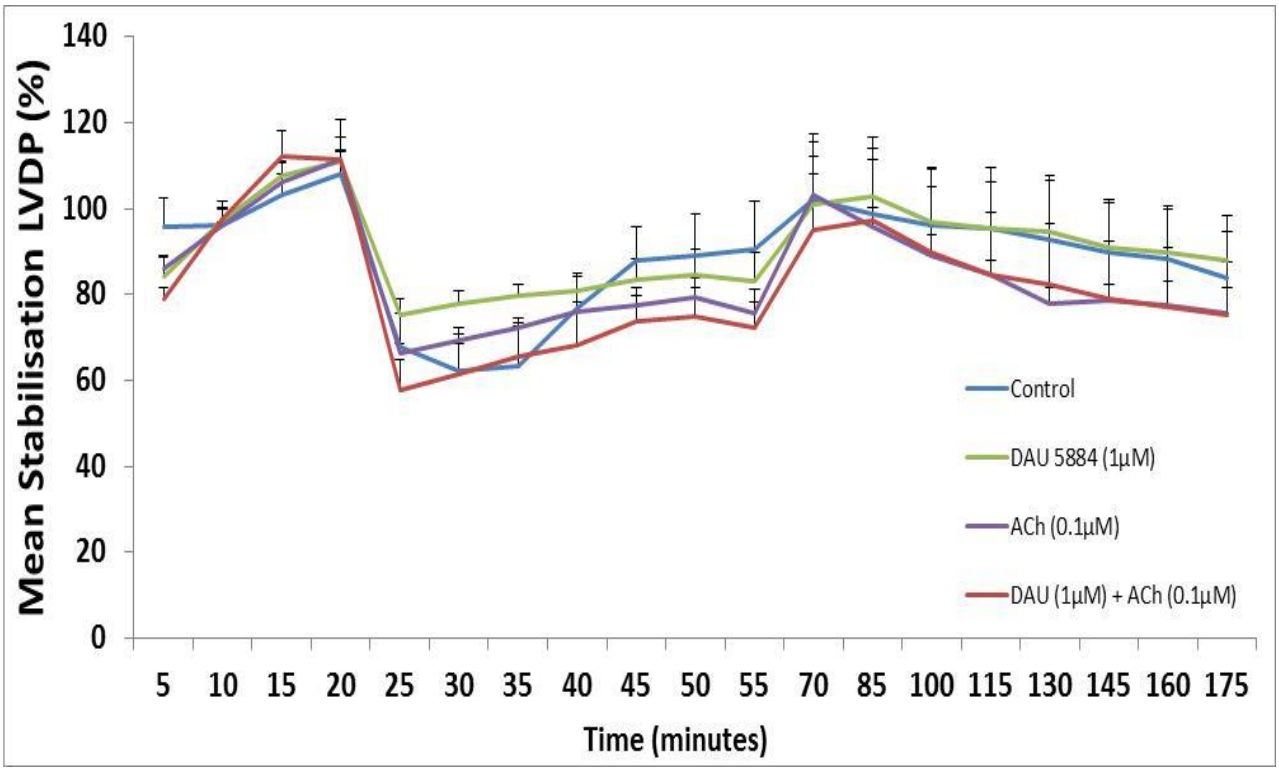

Figure 6.3: The effects of DAU 5884 (1µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on LVDP as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

The effects of DAU 5884 (1µM) ± ACh (0.1µM) treatment on the HR are shown in figure 6.4. The results showed that the DAU 5884 (1µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the heart rate as compared to the untreated control.

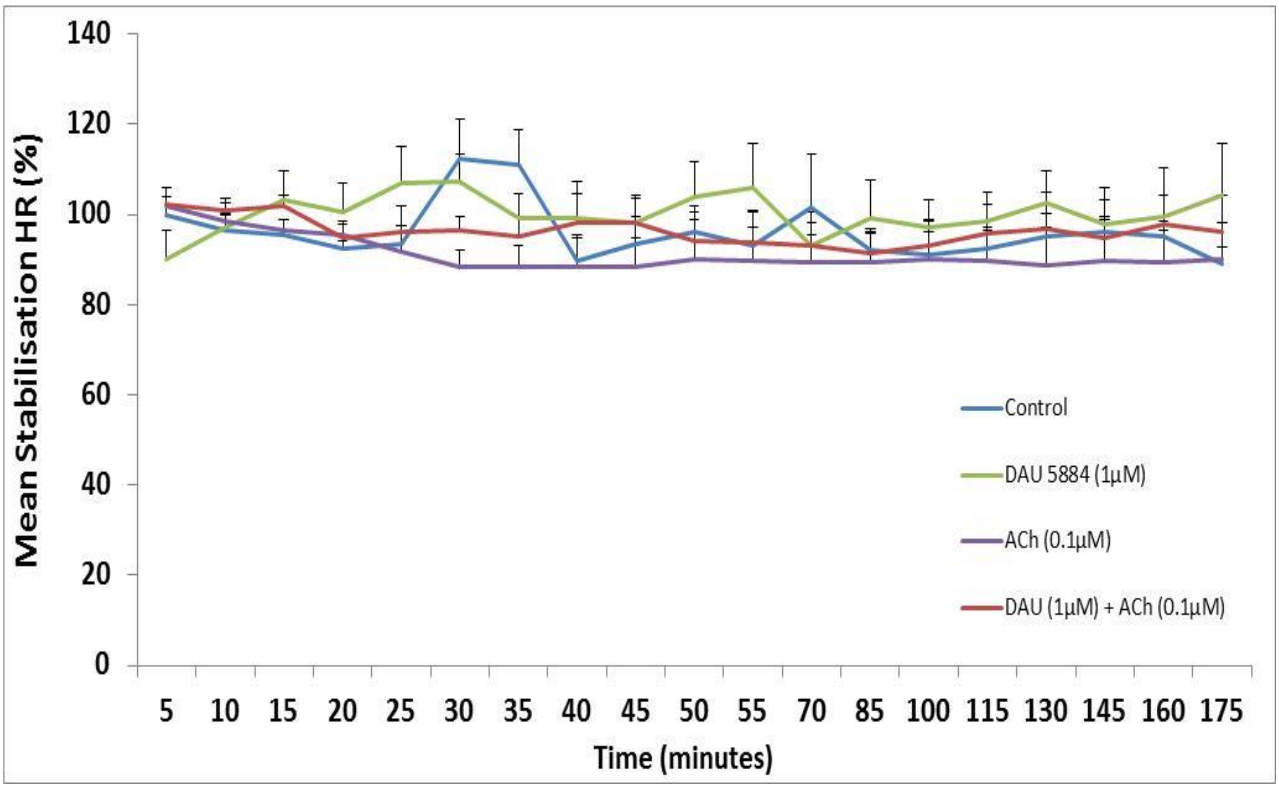

Figure 6.4: The effects of DAU 5884 (1µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on HR as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

CF was recorded by collecting the effluent for 1 minute at regular intervals; data presented are calculated, corrected for the heart weight, and plotted as a percentage of mean stabilisation. The effects of DAU 5884 (1µM) ± ACh (0.1µM) treatment on the coronary flow are shown in figure 6.5. The results showed that the DAU 5884 (1µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) treatment did not cause a significant change in the coronary flow as compared to the untreated control.

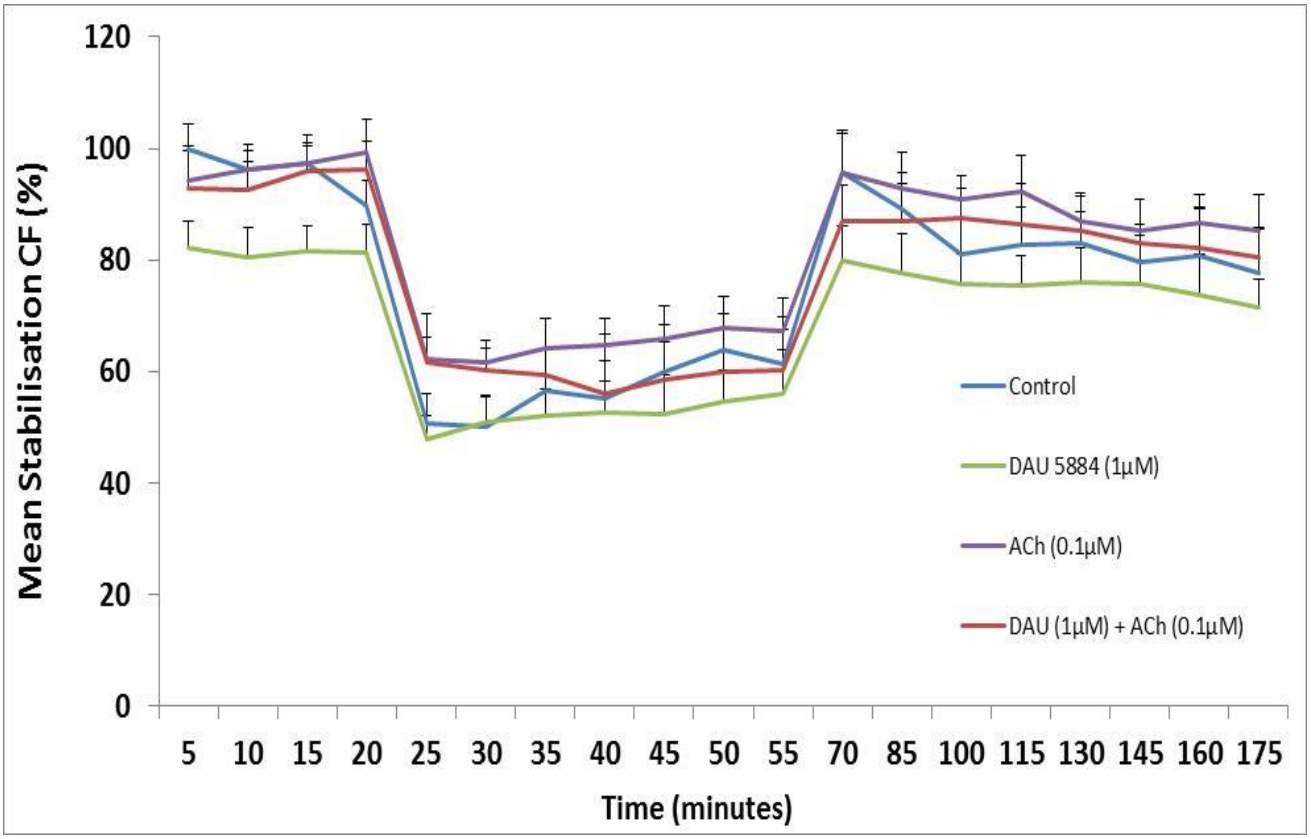

Figure 6.5: The effects of DAU 5884 (1µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on CF as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

6.3.4 The effects of DAU 5884 on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

Cardiac myocytes were isolated following the protocol mentioned in section 2.3.1 and were incubated with MTT in the dark for 2 hours to assess the cell viability. The reduction of MTT to formazan by mitochondrial dehydrogenase and corresponding colour change was indicative of the relative changes in myocytes survival and demonstrated the cell viability. The effects of the increasing concentration of DAU 5884 (0.03µM-3µM) on cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation were investigated. The DAU 5884 was added at the onset of re-oxygenation. The results (figure 6.6) showed that DAU 5884 (1µM and 3µM) led to

significantly reduced cell viability as compared to the control [75.32 ± 6.33 % (DAU 5884, 1µM) vs. 100 ± 0% (control) $p<0.05$, 73.27 ± 5.69 % (DAU 5884, 3µM) vs. 100 ± 0% (control) $p<0.05$]. The data are shown in table 6.2. Although DAU 5884 was solubilised in DMSO but due to time constraint a vehicle control was not performed.

Table 6.2: The effect of DAU 5884 (0.003µM-3µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	0.003µM	0.01µM	0.03µM	0.1µM	0.3µM	1µM	3µM
Cell Viability (%)	100	99.5	100	98.5	83.5	80.3	75.32	73.27
SEM	0	12.65	9.28	8.09	4.31	4.45	6.33	5.69

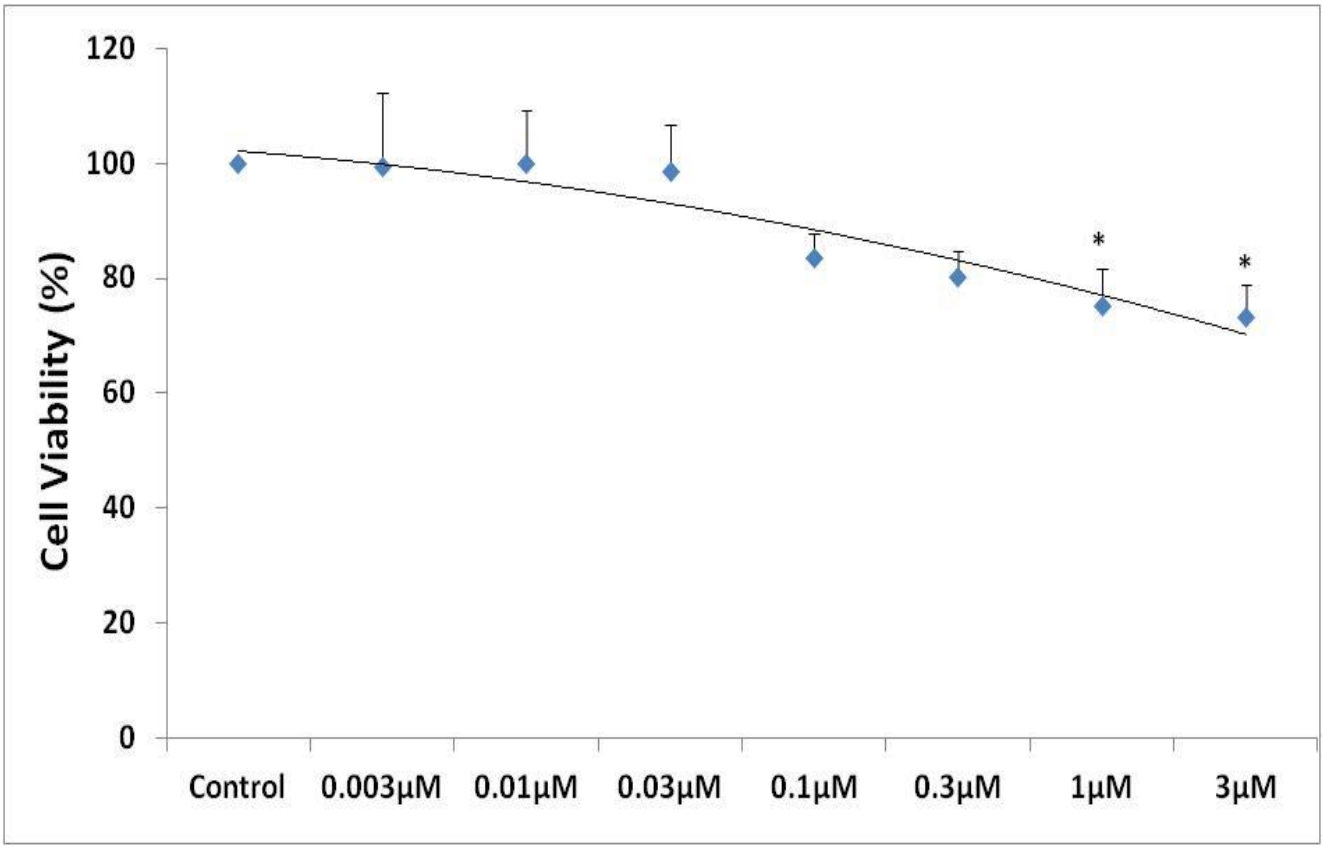

Figure 6.6: MTT analysis showing cell viability of cardiac myocytes undergoing 2 hours of hypoxia and 2 hours of re-oxygenation in response to increasing concentrations of DAU 5884 (0.03µM-3µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control.

6.3.5 The effects of DAU 5884 ± ACh on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

As the M_3 mAChR antagonist, DAU 5884 (1µM and 3µM) was shown to decrease the cell viability of cardiac myocytes (section 6.3.4), we investigated the effects of the natural mAChR agonist, ACh on the cardiac myocytes undergoing hypoxia and re-oxygenation. The results (figure 6.7) showed that treatement with ACh (0.1µM) alone did not significantly change the viability of cardiac myocytes (Figure 6.7). However, co-administration of DAU 5884 (1µM) with ACh (100nM) abrogated the damage caused by DAU 5884 (1µM) [86.25 ± 7.16 % (DAU 5884, 1µM + ACh, 0.1 µM) vs. 75.32 ± 6.33% (DAU 5884, 1µM) $p<0.05$]. The data are shown in table 6.3.

Table 6.3: The effect of DAU 5884 (1µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	DAU 5884 (1µM)	ACh (0.1µM)	DAU (1µM) + ACh (0.1µM)
Cell Viability (%)	100	75.32	92.62	86.25
SEM	0	6.33	3.16	7.16

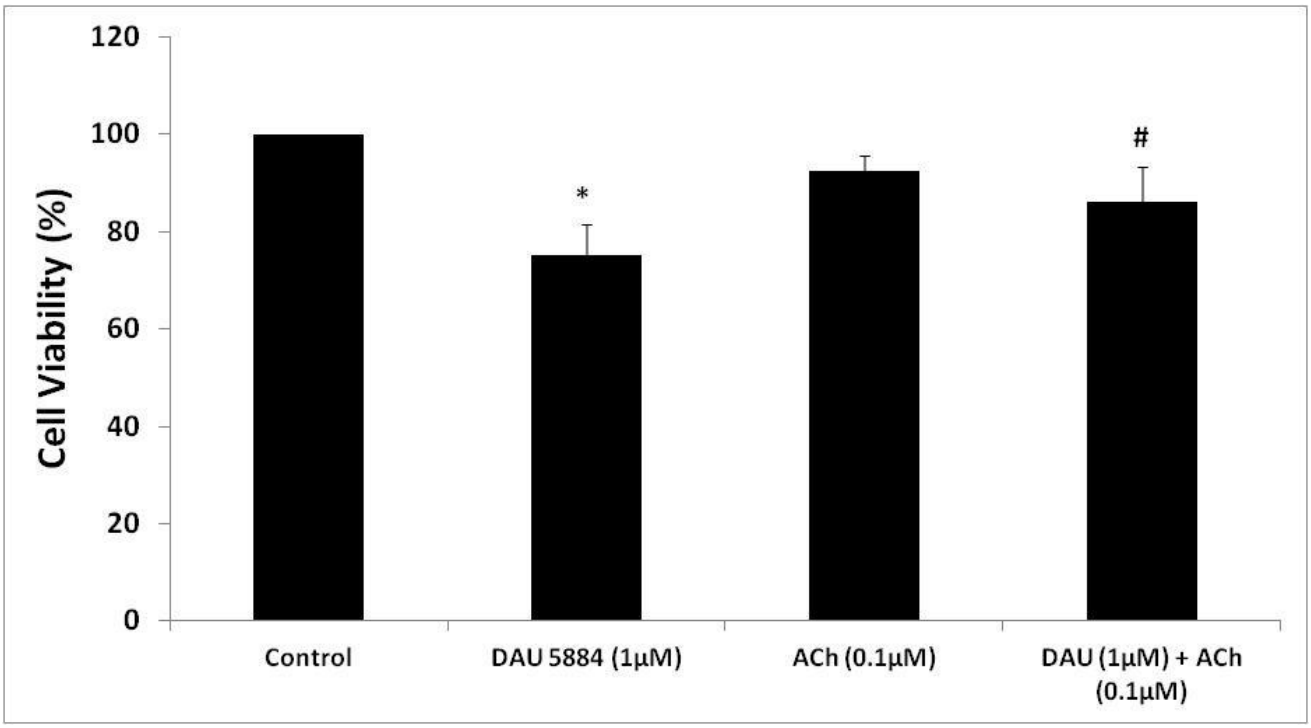

Figure 6.7: MTT analysis showing cell viability of cardiac myocytes in response to the treatment of DAU 5884 (1µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control and #p<0.05 vs. DAU 5884 (1µM).

6.3.6 The effects of drug treatment on the levels of signalling proteins as assessed by western blot analysis

To understand the molecular signalling mechanism via which DAU 5884 has shown to exacerbate myocardial ischaemia reperfusion injury (section 6.3.1) and decreased cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation (section 6.3.4), we performed western blot analysis. The effect of drug treatment on the levels of phosphorylated Akt, ERK and SAPK/JNK at 20 minutes into the reperfusion phase was investigated.

Results (figure 6.8) showed that DAU 5884 (1µM, 3µM) led to significantly reduced levels of phosphorylated Akt as compared to the control [73.5 ± 5.73 % (DAU 5884, 1µM) vs. 100 ± 0% (control), $p<0.05$, 66.1 ± 3.06 % (DAU 5884, 3µM) vs. 100 ± 0% (control), $p<0.01$]. However, administration of ACh (0.1µM) at 20 minutes into reperfusion caused a significant increase in p-

Akt levels as compared to the Control [205 ± 18.5 % (ACh, 0.1µM) vs. 100 ± 0% (control), $p<0.05$].

Table 6.4: The effect of DAU 5884 (1µM, 3µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU (3µM)	DAU (1µM)	ACh (0.1µM)	DAU (1µM) + ACh (0.1µM)
p-Akt/Total Akt (%)	100	66.16	73.5	205	88.15
SEM	0	3.06	5.73	18.5	9.62

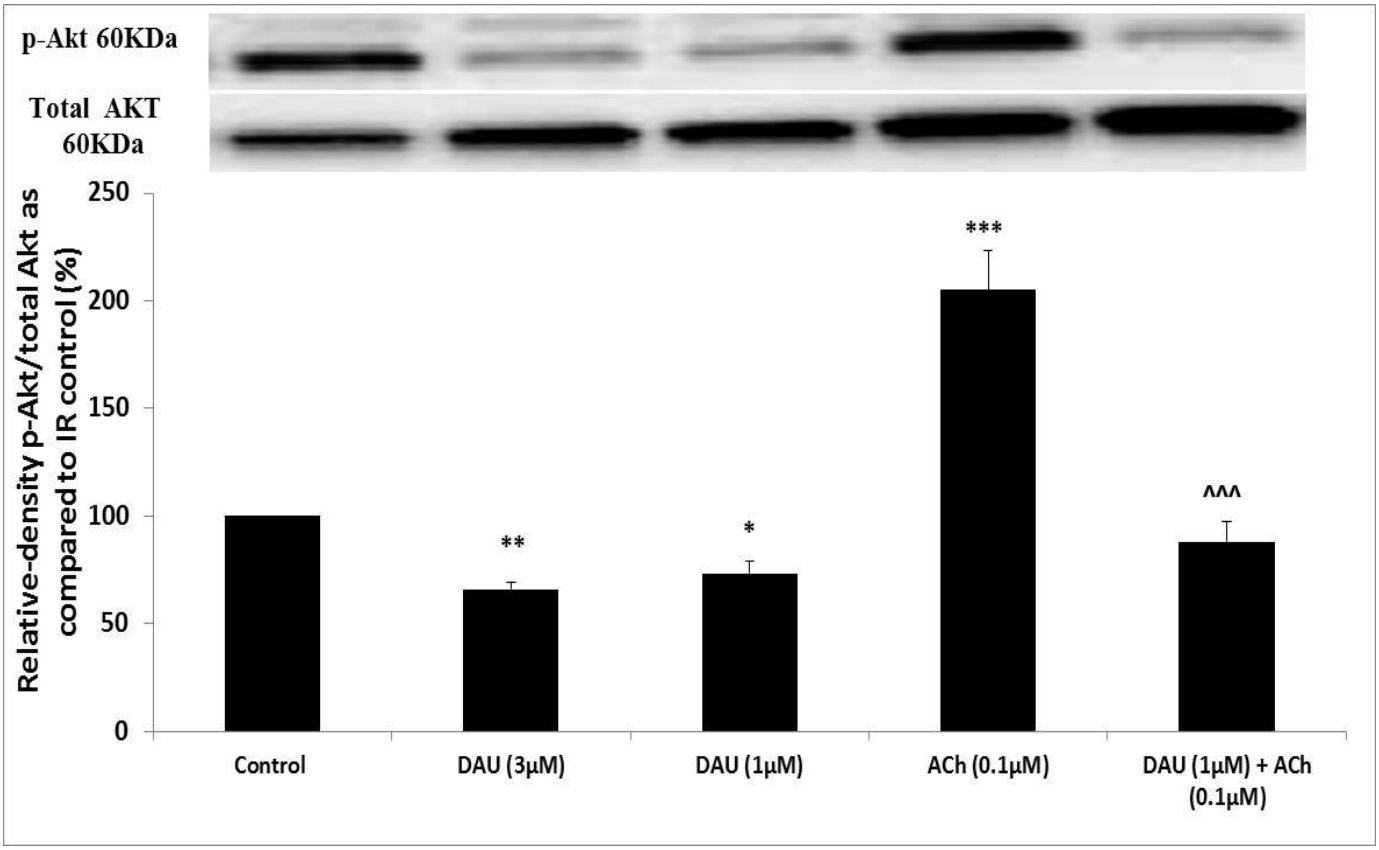

Figure 6.8: The effects of DAU 5884 (1µM, 3µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 and **p<0.01 vs. Control, ^^^ p<0.001 vs. ACh (0.1µM).

In addition, the results (figure 6.9) also showed that the M_3 antagonist, DAU 5884 (3µM) led to significantly reduced expression levels of phosphorylated ERK 1/2 as compared to the control

[65.22 ± 8.05 % (DAU 5884, 3µM) vs. 100 ± 0% (control), p<0.05]. The administration of ACh (0.1µM) at 20 minutes into reperfusion caused a significant increase in p-ERK 1/2 levels as compared to the Control [173.8 ± 41.2 % (ACh, 0.1µM) vs. 100 ± 0 % (control), p<0.05]. The data are shown in table 6.5.

Table 6.5: The effect of DAU 5884 (1µM, 3µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU (3µM)	DAU (1µM)	ACh (0.1µM)	DAU (1µM) + ACh (0.1µM)
p-ERK/Total ERK (%)	100	65.22	81.59	173.8	107.01
SEM	0	8.05	14.73	41.2	12.62

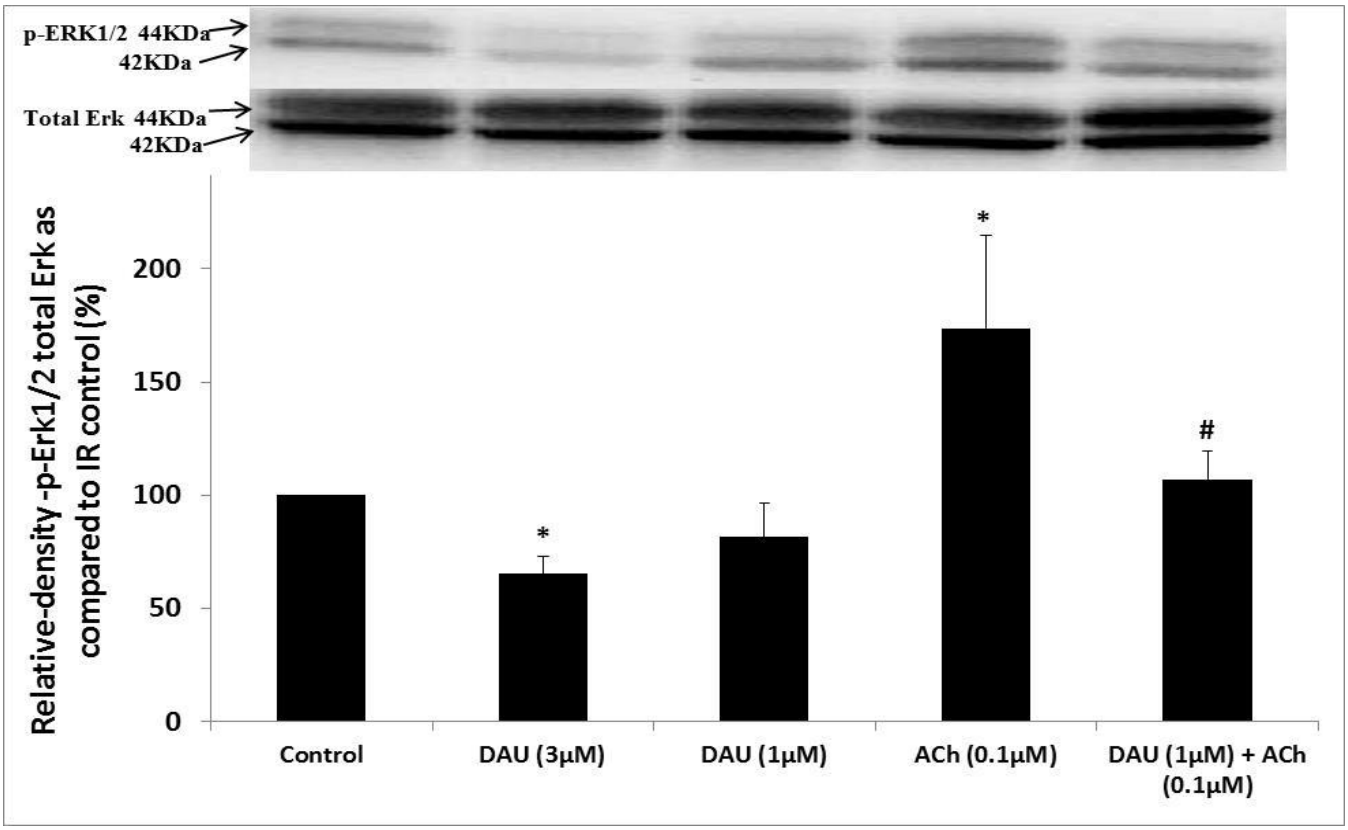

Figure 6.9: The effects of DAU 5884 (1µM, 3µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 vs. Control, # p<0.05 vs. DAU 5884 (1µM).

Western blot analyses also showed that DAU 5884 (1µM, 3µM) led to significantly increased p-SAPK/JNK levels as compared to the control [131.75 ± 14.73 % (DAU 5884, 1µM) vs. 100 ± 0% (control), $p<0.05$, 154.72 ± 8.06 % (DAU 5884, 3µM) vs. 100 ± 0% (control), $p<0.01$, (figure 6.10)]. In addition, ACh (0.1µM) led to significantly reduced p-SAPK/JNK levels as compared to the DAU 5884 (1µM) [100.19 ± 11.29 % (ACh, 0.1µM) vs. 131.75 ± 14.73 % (DAU 5884, 1µM), $p<0.05$]. Furthermore, the co-administration of DAU 5884 (1µM) with ACh (0.1µM) led to significantly reduced p-SAPK/JNK levels as compared to the increased levels observed by DAU 5884 (1µM) alone [95.14 ± 12.62 % (DAU 5884, 1µM + ACh, 0.1µM) vs. 131.75 ± 14.73% (DAU 5884, 1µM), $p<0.05$]. The data are shown in table 6.6.

Table 6.6: The effect of DAU 5884 (1µM, 3µM), ACh (0.1µM) and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU (3µM)	DAU (1µM)	ACh (0.1µM)	DAU (1µM) + ACh (0.1µM)
p-SAPK/JNK/ Total SAPK/JNK (%)	100	154.72	131.75	100.19	95.14
SEM	0	8.06	14.73	11.29	12.62

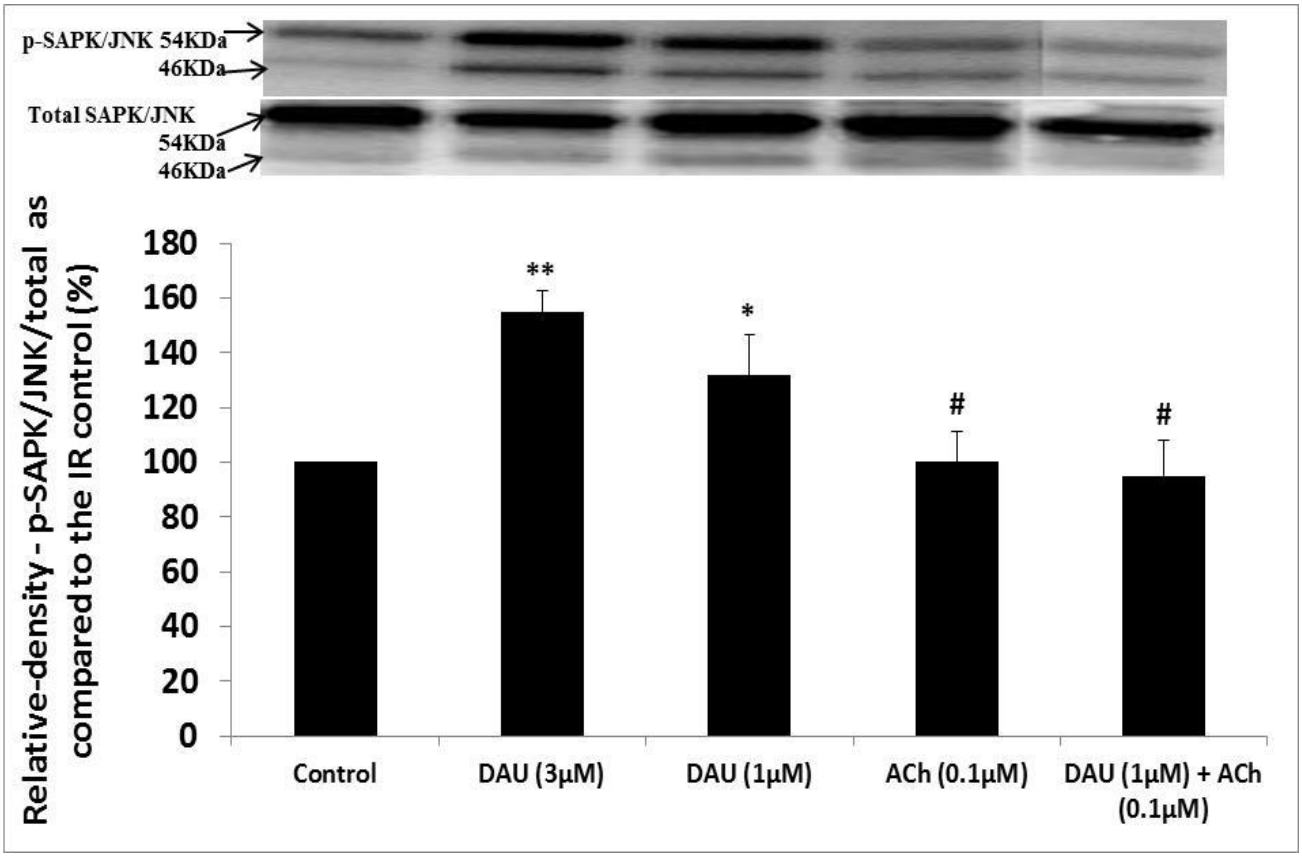

Figure 6.10: The effects of DAU 5884 (1µM, 3µM), ACh (0.1µM), and co-administration of DAU 5884 (1µM) with ACh (0.1µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 and **p<0.01 vs. Control, # p<0.05 vs. DAU 5884 (1µM).

6.4 Discussion

The current study indicates that the M_3 muscarinic receptor antagonist DAU 5884 significantly exacerbates myocardial injury in *ex vivo* conditions of simulated ischaemia- reperfusion in a dose dependent manner. This observation was further confirmed in isolated cardiac myocytes revealing a reduction in cell viability upon treatment with increasing concentrations of DAU 5884 during the re-oxygenation period. Furthermore, DAU 5884 also led to significantly decreased expression levels of cell survival proteins, p-Akt and p-ERK1/2 and increased the stress activated proteins, SAPK/JNK. Studies have shown that JNK is activated in response to environmental stresses including heat shock, UV radiation, osmotic shock and inflammatory

cytokines (Nishina *et al*. 2004). Evidence in support for activation of SAPK/JNK and its association with apoptosis and cell hypertrophy has been derived from various investigations in *in vitro* and *in vivo* systems. Wang *et al*. (1998) showed that activation of JNK in neonatal rat cardiac myocytes induced characteristic features of hypertrophy and also induced apoptosis. Furthermore, myocardial ischaemia-reperfusion has also been shown to activate JNK resulting in apoptosis (Yin *et al*. 1997). Activation of SAPK/JNK has also been shown to result in apoptotic death of primary neonatal rat cells (Luo *et al*. 1998). Kim *et al*. (2001) also showed that activation of SAPK/JNK leads to cell death and its inhibition suppresses cell death in human U937 leukaemia cells. Our results are in line with the previous findings suggest a key role of SAPK/JNK in the pathophysiology of cardiac injury in response to various conditions, including ischaemia and reperfusion injury.

This study also showed that the M_3 mAChR antagonist, DAU 5884 led to significantly decreased levels of Akt and ERK 1/2, and increased the ischaemia reperfusion injury and reduced cell viability of isolated cardiac myocytes. Roy *et al*. (2010) showed that inhibition of Akt and ERK 1/2 leads to activation of transcription factors that lead to cell cycle arrest and consequently apoptosis in human pancreatic cancer cells. Furthermore, Fan *et al*. (2014) also recently showed that the inhibition of Akt and ERK 1/2 leads to apoptotic cell death in *in vitro* and *in vivo* human lung adenocarcinoma cells. Our findings also suggest the importance of inhibition of Akt and ERK 1/2 in exacerbation of myocardial ischaemia reperfusion injury. To our knowledge, this is the first study to show the detrimental effects of the M_3 mAChR antagonist, DAU 5884, in the setting of myocardial ischaemia- reperfusion injury and oxidative stress induced by hypoxia and re-oxygenation.

In this study we also demonstrated that the mAChR agonist ACh administered at the onset of reperfusion protected the heart from the damage caused by ischaemia- reperfusion injury and also reversed the DAU 5884-induced damage to the myocardium (Figure 6.2). The protective properties of ACh are well documented (Critz *et al*. 2005, Zang *et al*. 2007, Li *et al*. 2011) including in conditions of myocardial ischaemia-reperfusion (Richard *et al*. 1995). ACh has been shown to reduce the infarct size in rats during ischaemia reperfusion injury (Richard *et al*. 1995). Our results therefore confirm previous studies that have also shown the myocardial protective effects of ACh during ischaemia reperfusion injury.

This study also showed that ACh protects the myocardium against ischaemia reperfusion injury via activation of the cell survival proteins, Akt and ERK 1/2. Akt and ERK 1/2 protein kinases are comprised of the RISK pathway which upon activation has been shown to reduce myocardial injury (Hausenloy *et al*. 2005). Our results showed that treatment with ACh significantly increased Akt and ERK levels, thereby activating the RISK pathway to reduce the myocardial ischaemia reperfusion injury. Our data therefore support the literature from previous findings that activation of Akt and ERK reduces myocardial reperfusion injury.

Interestingly, the co-administration of ACh with DAU 5884 has abrogated the injury observed with DAU5884 alone. Endogenous levels of ACh have been shown to be present in primary rat cardiac myocytes (Dolezal and Tucek 1983). As DAU 5884 significantly increased the myocardial injury in the absence of exogenous ACh, we thereby postulate that DAU 5884 could therefore be inhibiting endogenous levels of ACh acting on the M_3 subtype.

6.5 Conclusion

To our knowledge, this is the first study to show that the M_3 mAChR antagonist, DAU 5884 significantly exacerbates myocardial ischaemia- reperfusion injury via activating the stress activated proteins SAPK/JNK and inhibiting Akt and ERK 1/2 protein kinases. On the other hand, exogenous ACh has shown to reduce this injury by activating the pro-survival kinases Akt and ERK 1/2 and down-regulating levels of SAPK/JNK. Activation of Akt and ERK 1/2 has been shown to protect cardiac myocytes against ischaemia- reperfusion injury (Fujio *et al*. 2000) and our results confirm these previous findings.

Chapter Seven: The M_3 muscarinic acetylcholine receptor antagonist, DAU 5884 exacerbates myocardial injury via opening of the mitochondrial permeability transition pore

7.1 Introduction

In the previous chapter we showed that the M_3 muscarinic receptor antagonist DAU 5884 significantly exacerbates myocardial injury in *ex vivo* conditions of simulated ischaemia-reperfusion in a dose dependent manner via decreasing expression levels of cell survival proteins, p-Akt and p-ERK1/2, and increasing the stress activated proteins, SAPK/JNK. Mitochondrial dysfunction has been shown to be an underlying cause of ischaemia-reperfusion injury (Crompton *et al*. 1999). It is involved in mediating lethal permeability changes to initiate cell death and has also been shown to be involved in drug induced myocardial injury by our lab (Gharanei *et al*. 2013). As the opening of the MPTP is a critical determinant of cell death in the setting of ischaemia-reperfusion injury, the current study therefore investigated the effect of the M_3 muscarinic receptor antagonist DAU 5884 on the MPTP in isolated rat cardiac myocytes.

There are various factors that increase the opening probability of the MPTP such as ATP depletion, oxidative stress, and high mitochondrial calcium and inorganic phosphate load (Hausenloy *et al*. 2003). These factors are similar to those during post-ischaemic reperfusion and there is growing evidence that MPTP opening plays an important role in the transition from reversible to irreversible reperfusion injury. Opening of the MPTP has been shown to be a critical determinant of cell death in the setting of ischaemia reperfusion injury (Hausenloy *et al*. 2003). The role of MPTP in ischaemia reperfusion injury has already been discussed in detail in chapter 1.6.

Studies suggest that procedures including inhibition of the MPTP opening or increase in subsequent pore closure reduce reperfusion injury (Halestrap *et al*. 2004). This may be either via direct pharmacological agents inhibiting MPTP opening or through an indirect effect associated with a decrease in the factors responsible for MPTP opening such as oxidative stress and calcium overload. Pharmacological inhibition of the MPTP opening has been shown to reduce myocardial injury in ischaemia reperfusion heart models to protect the heart (Hausenloy *et al*. 2002). We have also previously shown in chapter 5 that the inhibition of the MPTP by reducing the depolarisation and hypercontracture time by CsA reduced the ischaemia reperfusion injury. Furthermore, the injury caused by the M_2 mAChR antagonist, AF-DX 116 was also abrogated. In the current study, we therefore aim to investigate the involvement of M_3 mAChR antagonist DAU 5884 in the presence and absence of the MPTP blocker CsA, on MPTP activity using isolated cardiac myocytes subjected to oxidative stress. We will also evaluate the effects of DAU 5884 in the absence and presence of CsA on signaling proteins including cell survival proteins such as Akt, ERK 1/2, and stress activated protein, SAPK/JNK.

7.2 Methods

7.2.1 Isolated perfused heart preparation

Male Sprague-Dawley rats were sacrificed (n=6) and the hearts were dissected as described in section 2.2. The hearts were mounted on the Langendorff system and perfused with KH buffer. LVDP, HR and CF were measured and recorded at regular intervals. The same procedure was followed as mentioned in sectioned 2.2 to calculate the percentage of infarct to risk ratio.

7.2.2 Langendorff protocol

The hearts were stabilised for 20 minutes and subjected to 35 minutes of ischaemia followed by reperfusion for 120 minutes. Haemodynamic variables were recorded at regular 5 min intervals during stabilisation and ischaemia and every 15 min post-reperfusion. The drugs were administered at the onset of, and throughout, reperfusion.

The hearts were randomly assigned to the following groups: a) hearts perfused with KH buffer alone with 30 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with DAU 5884 (1µM); c) hearts perfused with CsA (0.2µM); d) hearts perfused with co-administration of DAU 5884 (1µM) and CsA (0.2µM). CsA has been previously shown to inhibit MPTP opening at a concentration of 0.2µM (Gharanei *et al*. 2014), hence we used the same concentration with DAU 5884 (1µM) to investigate CsA induced protection against mAChR antagonist mediated injury.

7.2.3 MTT analysis of cell viability

Adult (3-4 months old) male Sprague-Dawley rats were sacrificed (n=4) and the hearts were mounted on a Langendorff apparatus as mentioned in section 2.3.1 to isolate cardiac myocytes. The procedure mentioned in 2.3.2 was followed for the isolated cardiac myocytes to undergo hypoxia and re-oxygenation. The drugs were administered at the start of re-oxygenation. The cells were randomly assigned to the following treatment groups: a) Cells not treated with drug and undergoing hypoxia for 2 hours and 2 hours of re-oxygenation (control); b) Cells treated with DAU 5884 (1µM) administered at the onset of re-oxygenation following 2 hours of hypoxia; c) Cells treated with CsA (0.2µM) for 2 hours administered at the onset of re-oxygenation following 2 hours of hypoxia; d) Cells treated with the co-administration of DAU

5884 (1µM) with CsA (0.2µM). The % cell viability of samples was calculated as mentioned in section 2.3.2.

7.2.4 Protocol to analyse MPTP opening

The protocol mentioned in section 2.4 was followed to measure the MPTP opening. Briefly, cardiac myocytes were isolated as mentioned in section 2.3.1. The viability of the cells was assessed by a light microscope and the cell isolation yielding a viability of 65 % or more was used in the studies. The cardiac myocytes were plated onto glass cover slips coated with laminin to allow cell adhesion and incubated in microscopy buffer containing TMRM. TMRM is positively charged and selectively localises in the negatively charged inner-membrane of the mitochondrion in a membrane potential-dependent manner (Perry *et al*. 2011). The cells were then incubated in the absence or presence of drugs for 10 minutes before being placed on the confocal microscope. Cells were randomly assigned to the following drug treatment groups: a) Cells not treated with drug (control); b) Cells treated with DAU 5884 (1µM); c) Cells treated CsA (0.2µM); d) Cells treated with a combination of DAU 5884 (1µM) with CsA (0.2µM).

The cells were viewed under a Zeiss 510 CLSM confocal microscope equipped with 20x objective lens (NA 1.3) and a heated stage. A 585-nm long pass filter allowed detection of TMRM. Laser stimulation via the 543-nm emission line of a HeNe laser was used to induce oxidative stress. Recording and analysis was facilitated by use of the Zeiss software package, LSM 2.8. The time of cells to undergo both depolarisation and hypercontracture was recorded.

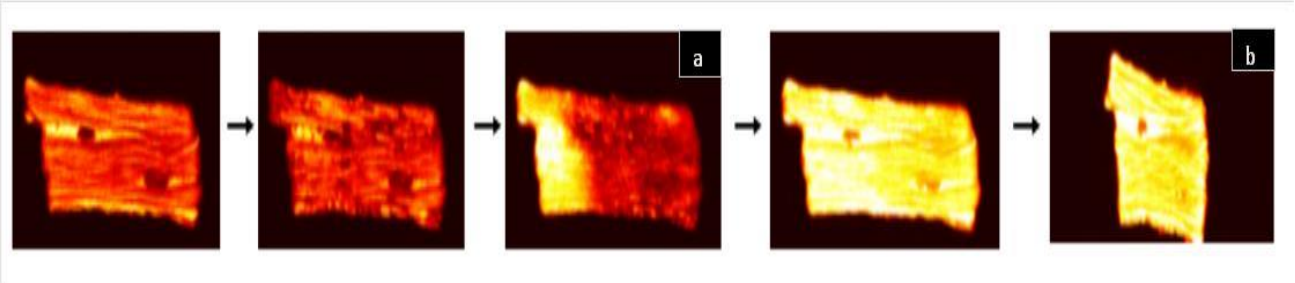

Figure 7.1: Confocal image of an adult rat ventricular myocyte undergoing (a) depolarisation and (b) hypercontracture

7.2.5 Western blot analysis of the isolated perfused heart tissue following drug treatment

Western blot analyses were carried out as mentioned in section 2.5. The samples were randomly assigned in the following experimental groups: a) hearts perfused with KH buffer alone with 35 minutes of ischaemia and 120 minutes of reperfusion (control); b) hearts perfused with AF-DX 116 at a concentration range of 0.1µM-3µM; c) hearts perfused with ACh (0.1µM); d) hearts perfused with co-administration of AF-DX 116 (1µM) and ACh (0.1µM). After separation, the proteins were transferred onto the PVDF membrane and probed for the phosphorylated and the total form of Akt (Ser_{473}), ERK 1/2 (Thr_{202}/ Tyr_{204}) and SAPK/JNK (Thr_{183}/Tyr_{185}). The relative changes in the phosphorylated protein levels were calculated and corrected for differences in protein loading as established by probing for total form of Akt, ERK 1/2 and SAPK/JNK into 20 minutes of reperfusion phase.

7.2.6 Statistical analysis

All values were expressed as mean ± SEM. Infarct size and cell viability were tested for group differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Haemodynamics were assessed for statistical difference using two way ANOVA with Fishers post hoc tests using SPSS 12. The time taken to depolarisation and hypercontracture were tested for group

differences using one way ANOVA with Fishers post hoc tests using SPSS 12. Differences were considered significant at $P \leq 0.05$.

7.3 Results

7.3.1 The effects of CsA ± DAU 5884 on the infarct size from the Langendorff experiments undergoing ischaemia reperfusion injury

The effects of DAU 5884, in the absence and presence of the MPTP blocker CsA, on the infarct size of the hearts following ischaemia reperfusion injury were investigated. The results (figure 7.2) showed that hearts treated with CsA (0.2µM) significantly decreased the infarct size as compared to the untreated control [36.5 ± 1.99 % (CsA, 0.2µM) vs. 47.21 ± 2.1 % (control) $p<0.001$]. As also mentioned in section 6.3.1, DAU 5884 (1µM) significantly increased the infarct size as compared to the control [67.8 ± 1.61 % (DAU 5884, 1µM) vs. 47.21 ± 2.1 % (control) $p<0.001$]. Interestingly, the co-administration of CsA (0.2µM) with DAU 5884 (1µM) abrogated the injury observed with DAU 5884 alone [55.5 ± 1.52 % (CsA, 0.2µM, + DAU 5884, 1µM) vs. 67.8 ± 1.61 % (DAU 5884, 1µM) $p<0.01$]. The data are shown in table 7.1.

Table 7.1: The effect of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the infarct size to risk ratio as compared to the control and the relative SEM values (n=6).

Group	Control	DAU (1µM)	CsA (0.2µM)	CsA (0.2µM) + DAU (1µM)
Infarct size (%)	47	67.8	36.5	55.5
SEM	2.1	1.61	1.99	1.52

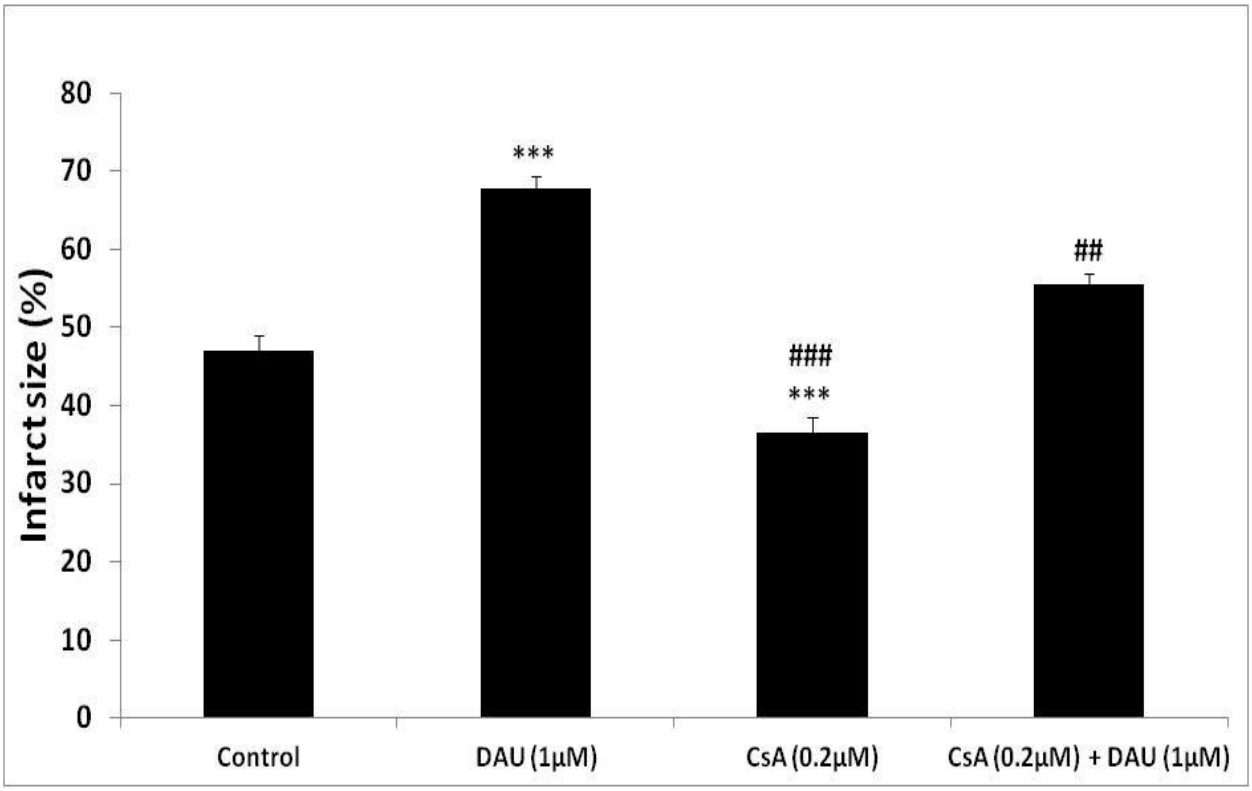

Figure 7.2: The effects of no drug treatment (control), DAU 5884 (1µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on infarct size to risk ratio in the whole heart Langendorff model of ischaemia reperfusion injury. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. Control. ##p<0.01 and ###p<0.001 vs. DAU 5884 (1µM).

7.3.2 The effects of CsA ± DAU 5884 on the haemodynamics of the heart

The haemodynamics including the LVDP, HR and CF of the hearts from the Langendorff model were recorded and measured. LVDP was calculated as the difference between systolic pressure and the diastolic pressure and presented as a percentage of mean stabilisation. The effects of CsA (0.2µM) ± DAU 5884 (1µM) treatment on the LVDP are shown in figure 7.3. The results showed that the DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the LVDP as compared to the untreated control.

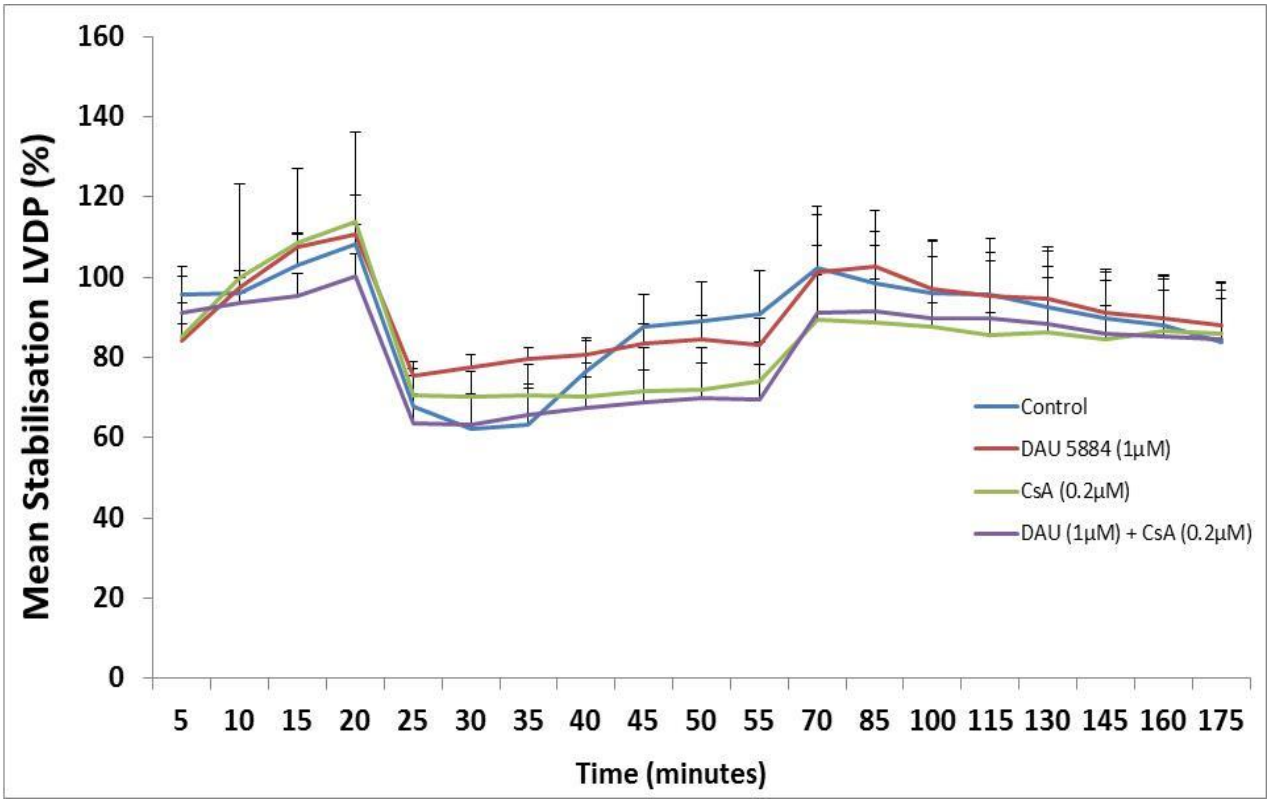

Figure 7.3: The effects of DAU 5884 (1µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on LVDP as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

The effects of DAU 5884 (1µM) ± CsA (0.2µM) treatment on the HR are shown in figure 7.4. The results showed that the DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the HR as compared to the untreated control.

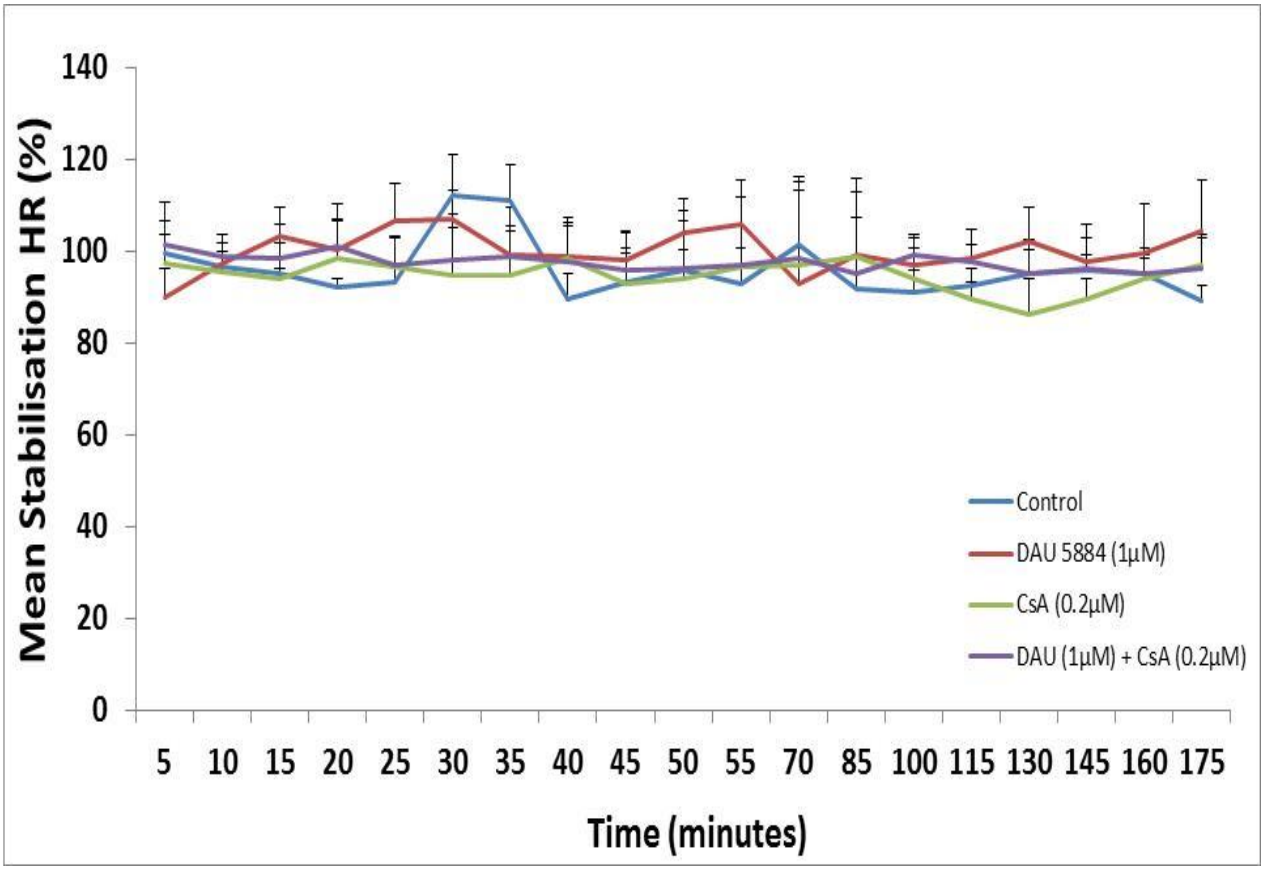

Figure 7.4: The effects of DAU 5884 (1µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on HR as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

CF was recorded by collecting the effluent for 1 minute at regular intervals; data presented are calculated, corrected for the heart weight, and plotted as a percentage of mean stabilisation. The effects of DAU 5884 (1µM) ± CsA (0.2µM) treatment on the CF are shown in figure 7.5. The results showed that the DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) treatment did not cause a significant change in the coronary flow as compared to the untreated control.

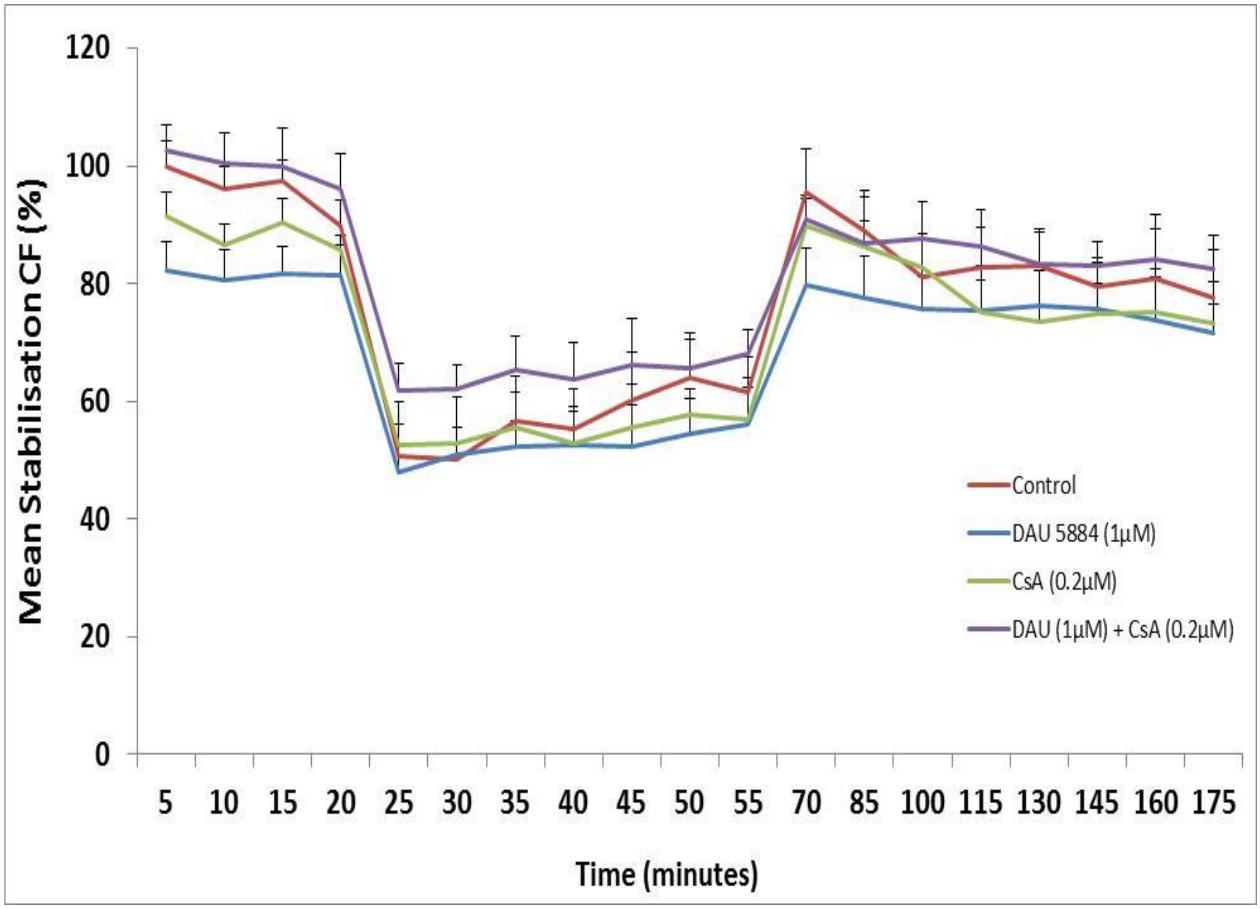

Figure 7.5: The effects of DAU 5884 (1µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on coronary flow as a percentage of mean stabilisation in rat hearts subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion. Drugs were added at the onset of reperfusion. Results are expressed as mean ± SEM (n=6).

7.3.3 The effects of DAU 5884 ± CsA on the viability of isolated cardiac myoctes under hypoxia and re-oxygenation

The effects of the MPTP blocker CsA, in the presence and absence of the M_3 mAChR antagonist DAU 5884, on the cardiac myocytes undergoing hypoxia and re-oxygenation were investigated. The results showed that the administration of DAU 5884 (1µM) at the onset of re-oxygenation led to significantly decreased cell viability as compared to the control [65.78 ± 9.27% (DAU 5884 1µM) vs. 100 ± 0% (Control)]. Treatment with CsA (0.2µM) alone did not significantly change the viability of cardiac myocytes (figure 7.6). However, co-administration of DAU 5884

(1µM) with CsA (0.2µM) abrogated the damage caused by DAU 5884 (1µM) alone [79.75 ± 6.77 % (DAU 5884, 1µM + CsA, 0.2µM) vs. 65.78 ± 9.27 % (DAU 5884, 1µM) $p<0.05$. The data shown are table 7.2.

Table 7.2: The effect of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the cell viability of cardiac myocytes undergoing hypoxia and re-oxygenation and the relative SEM values (n=4).

Group	Control	DAU (1µM)	CsA (0.2µM)	DAU (1µM) + CsA (0.2µM)
Cell Viability (%)	100	65.78	97.75	79.75
SEM	0	9.27	4.16	6.77

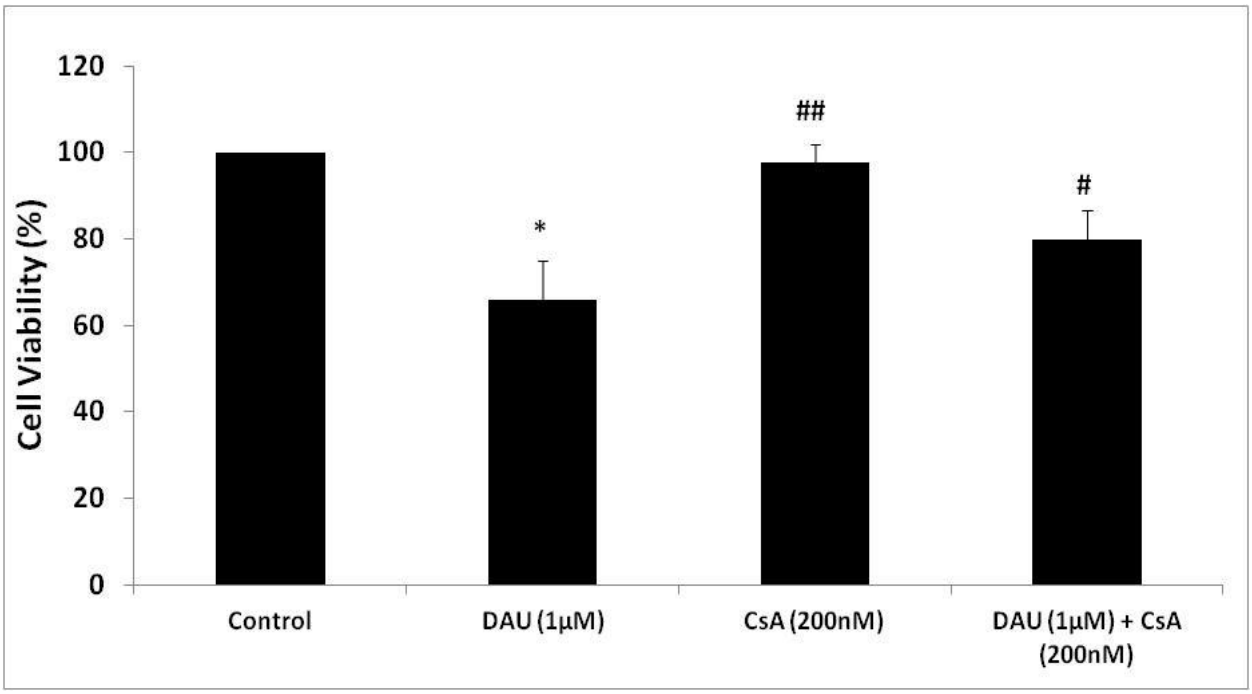

Figure 7.6: MTT analysis showing cell viability of cardiac myocytes in response to the treatment of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM). Drugs were added at the onset of re-oxygenation. Results are expressed as mean ± SEM (n=4). *p<0.05 vs. Control and #p<0.05 and ##p<0.01 vs. DAU 5884 (1µM).

7.3.4 The effects of drug treatment on laser-induced oxidative stress in cardiac myocytes

Laser induced oxidative stress initiates mitochondrial depolarisation which indicates MPTP opening. Laser stimulation initiates photodecomposition of TMRM thus generating mitochondrial reactive oxygen species, leading to disruption of the mitochondrial membrane. Depolarisation begins as a wave of increased cytosolic TMRM at one end of the cell and propagates throughout the cell (Hausenloy *et al*; 2003). Continued oxidative stress leads to hypercontracture of the cell which signifies ATP depletion.

The effects of the M_3 mAChR antagonist DAU 5884 on laser-induced oxidative stress in cardiac myocytes were investigated. The results (figure 7.7) showed that DAU 5884 (1µM) significantly reduced the depolarisation time as compared to the control [185.5 ± 12.5 sec (DAU 5884, 1µM) vs. 250.5 ± 12.6 sec (control) $p<0.001$]. Interestingly, CsA (0.2µM) treatment alone led to a significantly increased depolarisation time as compared to the control [317.5 ± 13.3 sec (CsA, 0.2µM) vs. 250.5 ± 12.6 sec (control) $p<0.001$]. Co-administration of CsA (0.2µM) with DAU 5884 (1µM) led to a significantly increased time to depolarisation as compared to DAU 5884 (1µM) alone [250.8 ± 10.1 sec (CsA, 0.2µM + DAU 5884, 1µM) vs. 185.5 ± 12.5 sec (DAU 5884, 1µM) $p<0.01$. The data are shown in table 7.3.

Table 7.3: The effect of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on hypercontracture time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6).

Group	Control	DAU 5884 (1µM)	CsA (0.2µM)	DAU (1µM) + CsA (0.2µM)
Mean depolarisation time (sec)	250.5	185.5	317.5	250.8
SEM	12.6	12.5	13.3	10.1

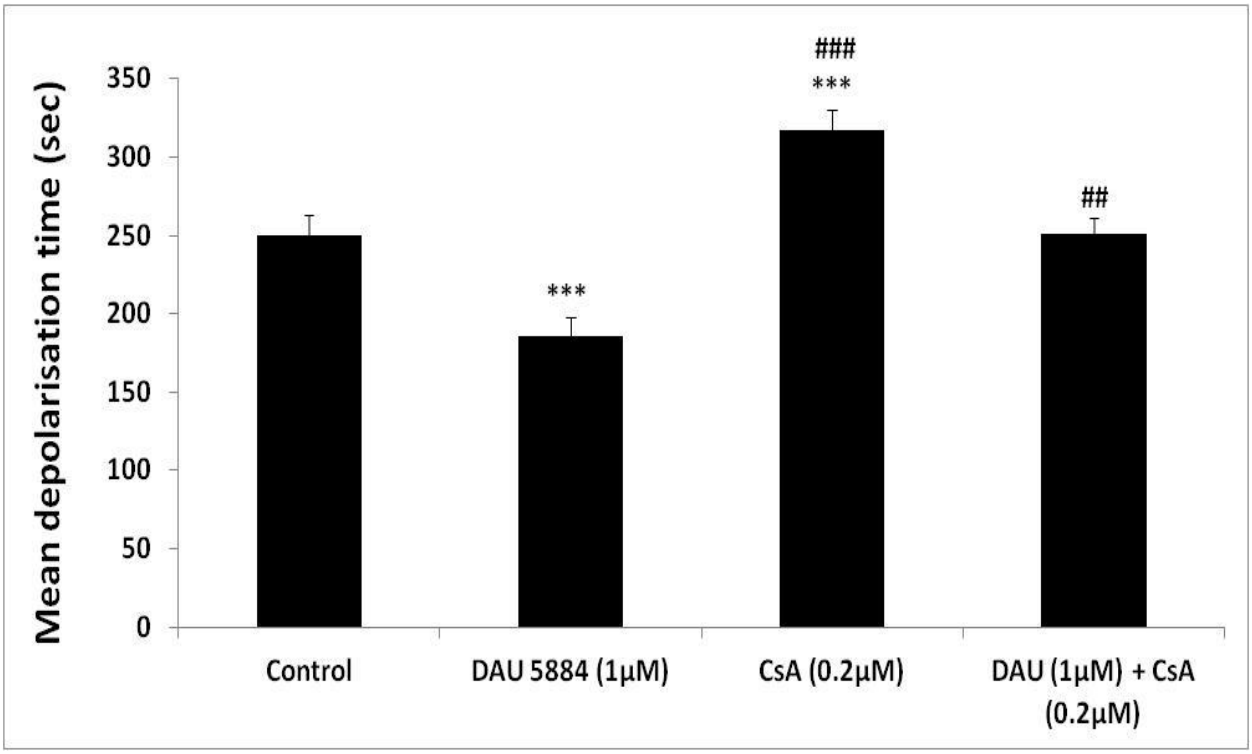

Figure 7.7: The effects of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on depolarisation time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. control, ##p<0.01 and ### = p<0.001 vs. DAU 5884 (1µM).

DAU 5884 (1µM) treatment of adult rat cardiac myocytes also led to a significantly reduced hypercontracture time as compared to the control [529 ± 44.7 sec (DAU 5884, 1µM) vs. 741.7 ± 21.5 sec (control) $p<0.001$, (figure 7.8)]. Furthermore, CsA (0.2µM) treatment alone led to a significantly increased hypercontracture time as compared to the control [873.3 ± 22.9 sec (CsA, 0.2µM) vs. 741.7 ± 21.5 sec (control) $p<0.001$]. Co-administration of CsA (0.2µM) with DAU 5884 (1µM) led to a significantly increased time to hypercontracture as compared to DAU 5884 (1µM) alone [706 ± 26.2 sec (DAU 5884, 1µM + CsA, 0.2µM) vs. 529 ± 44.7 sec (DAU 5884, 1µM) $p<0.001$]. The data are shown in table 7.4.

Table 7.4: The effect of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on hypercontracture time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6).

Group	Control	DAU 5884 (1µM)	CsA (0.2µM)	DAU (1µM) + CsA (0.2µM)
Mean hypercontracture time (sec)	741.7	529	873.3	706
SEM	21.5	44.7	22.9	26.2

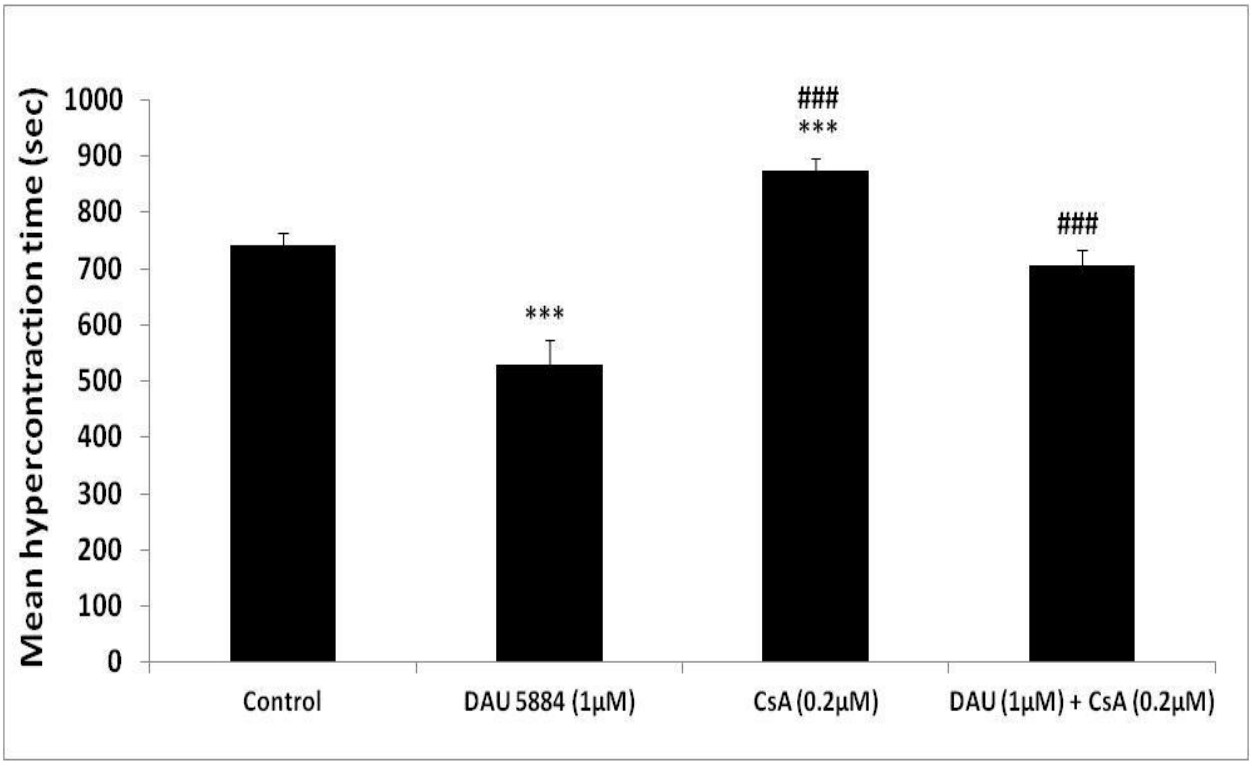

Figure 7.8: The effects of DAU 5884 (1µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on hypercontracture time in isolated rat cardiac myocytes in an oxidative stress model. Results are expressed as mean ± SEM (n=6). ***p<0.001 vs. control, ### = p<0.001 vs. DAU 5884 (1µM).

7.3.5 The effects of drug treatment on the levels of signalling proteins as assessed by western blot analysis

To understand the molecular signalling mechanism via which DAU 5884 exacerbates myocardial ischaemia reperfusion injury, and CsA reduced myocardial ischaemia reperfusion injury (section 7.3.1), we performed western blot analysis. The effect of drug treatment on the levels of

phosphorylated Akt, ERK and SAPK/JNK at 20 minutes into the reperfusion phase was investigated.

The results (figure 7.9) showed that DAU 5884 (1µM, 3µM) led to significantly reduced levels of phosphorylated Akt as compared to the control [73.5 ± 5.7 sec (DAU 5884, 1µM) vs. 100 ± 0 sec (control), $p<0.05$, 66.2 ± 3.1 sec (DAU 5884, 3µM) vs. 100 ± 0 sec (control), $p<0.01$]. The administration of CsA (0.2µM) at 20 minutes into reperfusion caused a significant increase in p-Akt levels as compared to the Control [150.9 ± 17.5 sec (CsA, 0.2µM) vs. 100 ± 0 sec (control), $p<0.01$]. Furthermore, the co-administration of DAU 5884 (1µM) with CsA (0.2µM) led to significantly lower expression than was observed with CsA (0.2µM) alone [63.6 ± 10.3 sec (DAU, 1µM + CsA, 0.2µM) vs. 150.9 ± 17.5 sec (CsA, 0.2µM), $p<0.001$].

Table 7.5: The effect of DAU 5884 (1µM, 3µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU 5884 (3µM)	DAU 5884 (1µM)	CsA (0.2µM)	DAU (1µM)+ CsA(0.2µM)
p-Akt/Total Akt (%)	100	66.2	73.5	150.9	63.6
SEM	0	3.1	5.7	17.5	10.3

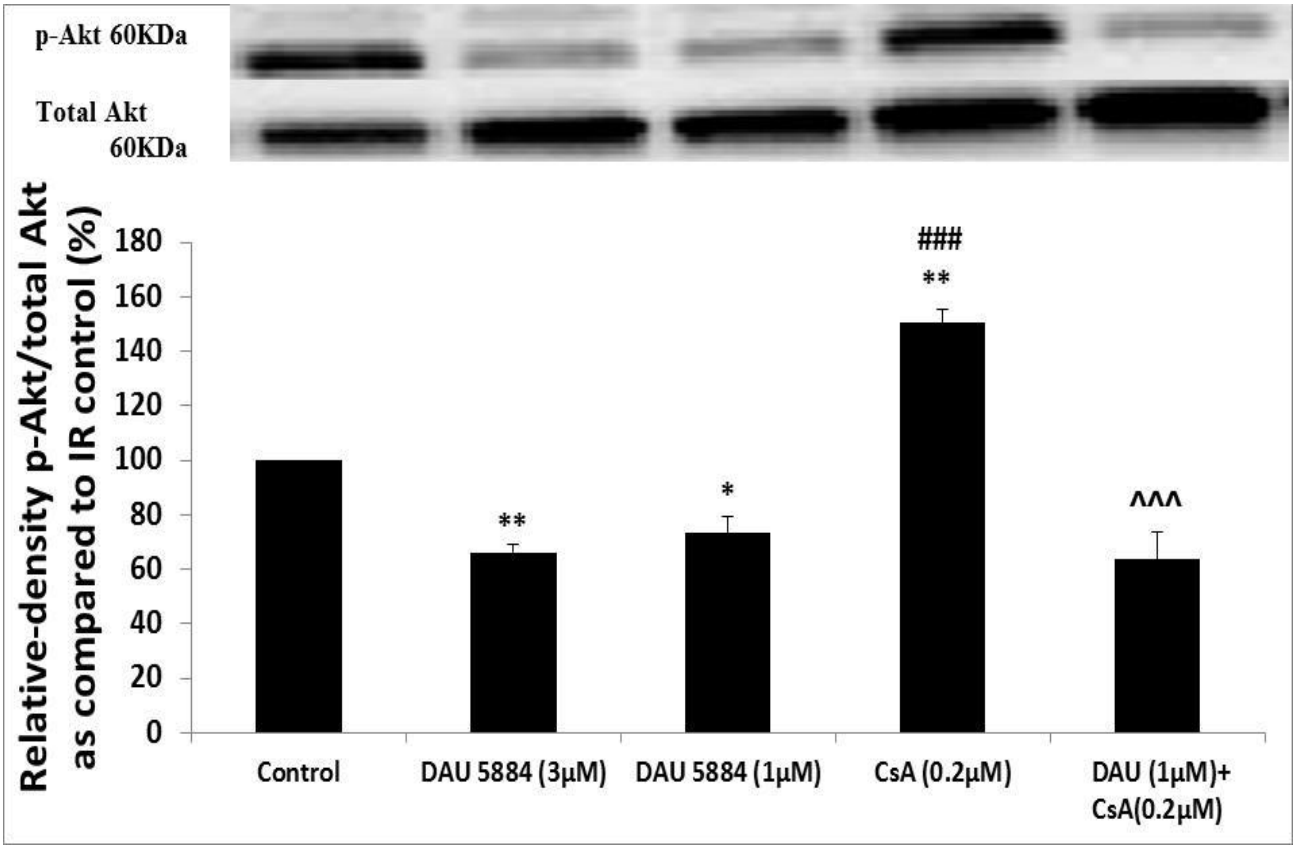

Figure 7.9: The effects of DAU 5884 (1μM, 3 μM), CsA (0.2μM), and co-administration of DAU 5884 (1μM) with CsA (0.2μM) on the levels of phosphorylated Akt at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 and **p<0.01 vs. Control, ###p<0.001 vs. DAU 5884 (1μM), ^^^p<0.001 vs. CsA (0.2μM).

In addition, the results also showed that the M_3 antagonist DAU 5884 (3μM) significantly reduced the expression levels of phosphorylated ERK 1/2 as compared to the control (figure 7.10). The administration of CsA (0.2μM) at 20 minutes into reperfusion caused a significant increase in p-ERK 1/2 levels as compared to the control [160.5 ± 10.8 % (CsA, 0.2μM) vs. 100 ± 0 % (Control), $p<0.01$]. Furthermore, co-administration of DAU 5884 (1μM) with CsA (0.2μM) led to significantly lower expression than was observed with CsA (0.2μM) alone [102.3 ± 10.2 % (DAU, 1μM + CsA, 0.2μM) vs. 160.5 ± 10.8 % (CsA, 0.2μM)]. The data are shown in table 7.6.

Table 7.6: The effect of DAU 5884 (1µM, 3µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU (3µM)	DAU (1µM)	CsA (0.2µM)	DAU (1µM)+ CsA(0.2µM)
p-ERK/Total ERK (%)	100	65.2	81.6	160.5	102.3
SEM	0	8.1	14.7	10.8	10.2

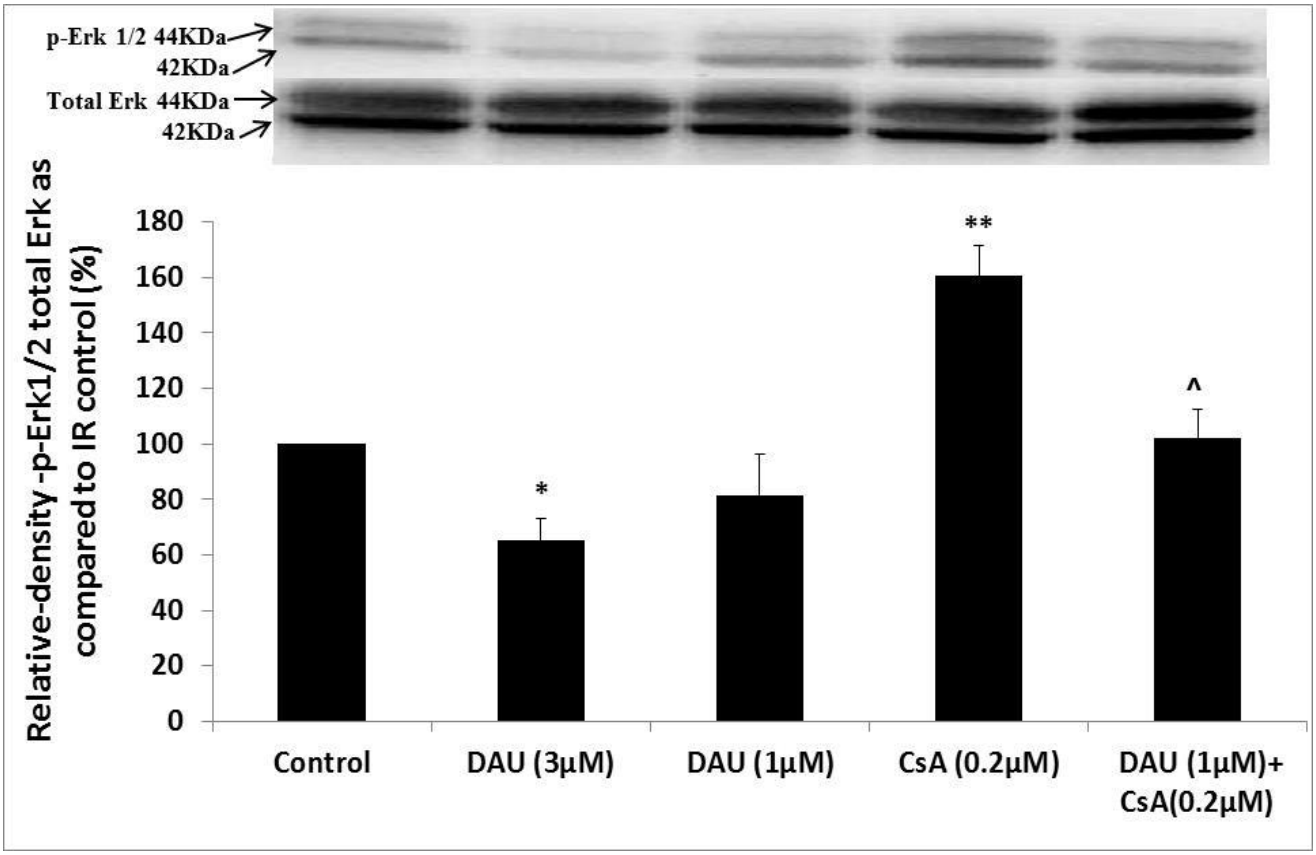

Figure 7.10: The effects of DAU 5884 (1µM, 3µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the levels of phosphorylated ERK 1/2 at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *p<0.05 and **p<0.001 vs. Control, ^ p<0.05 vs. CsA (0.2µM).

Western blot analyses also showed that DAU 5884 (1µM, 3µM) led to significantly increased p-SAPK/JNK levels as compared to the control [131.7 ± 14.7 % (DAU 5884, 1µM) vs. 100 ± 0 % (control), $p<0.05$, 154.7 ± 8.1 % (DAU 5884, 3µM) vs. 100 ± 0 % (control), $p<0.01$, figure 7.11]. In addition, CsA (0.2µM) led to significantly reduced p-SAPK/JNK levels as compared to the control [66.6 ± 6.2 % (CsA, 0.2µM) vs. 100 ± 0 % (control), $p<0.01$]. Furthermore, the co-

administration of DAU 5884 (1µM) with CsA (0.2µM) led to significantly increased p-SAPK/JNK levels as compared to the decreased levels observed with CsA (0.2µM) alone [103.2 ± 12.3 % (DAU 5884, 1µM + CsA, 0.2µM) vs. 66.6 ± 6.2 % (CsA, 0.2µM), $p<0.05$]. The data are shown in table 7.7.

Table 7.7: The effect of DAU 5884 (1µM, 3µM), CsA (0.2µM) and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase and the relative SEM values.

Group	Control	DAU (3µM)	DAU (1µM)	DAU (1µM) + CsA (0.2µM)	CsA (0.2µM)
p-SAPK/JNK/Total SAPK/JNK (%)	100	154.7	131.7	103.2	66.6
SEM	0	8.1	14.7	12.3	6.2

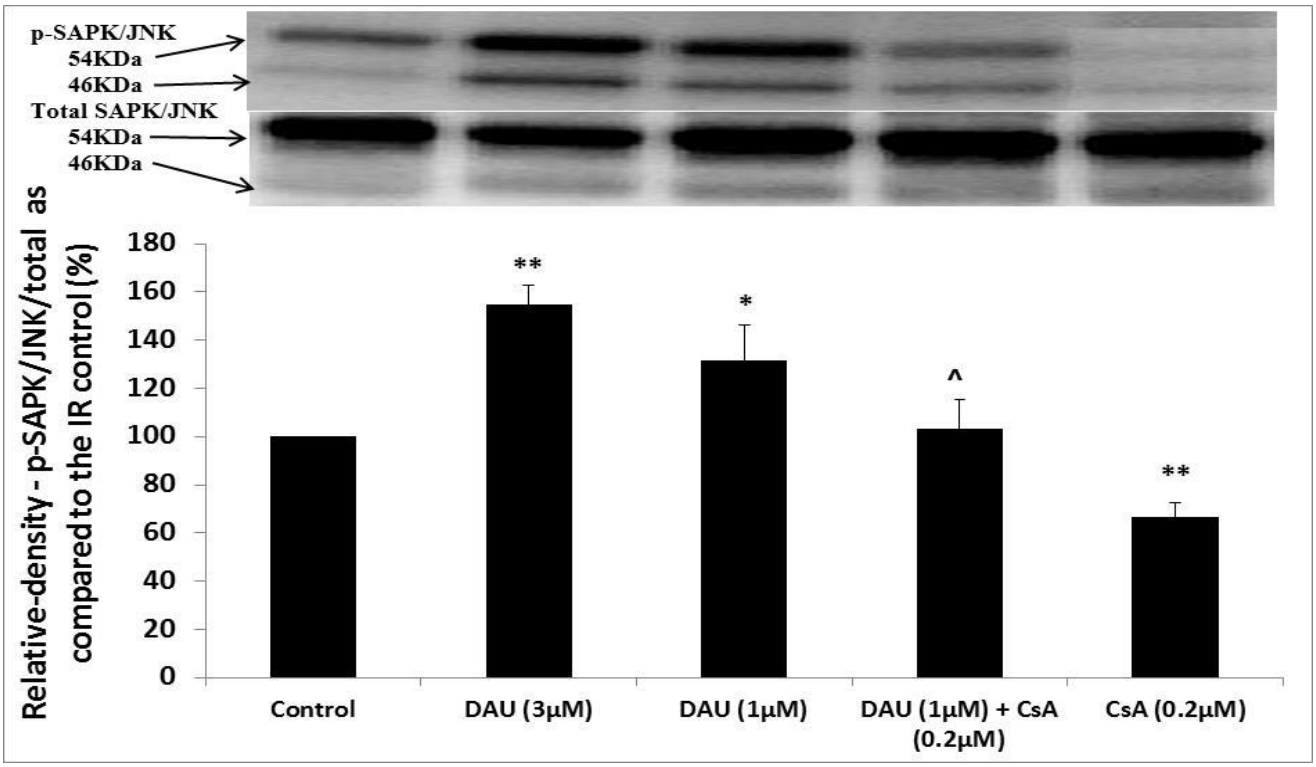

Figure 7.11: The effects of DAU 5884 (1µM, 3µM), CsA (0.2µM), and co-administration of DAU 5884 (1µM) with CsA (0.2µM) on the levels of phosphorylated SAPK/JNK at 20 minutes into the reperfusion phase. Results are expressed as mean ± SEM (n=3). *$p<0.05$ and **$p<0.001$ vs. Control, ^ $p<0.05$ vs. CsA (0.2µM).

7.4 Discussion

Functional affinities of a variety of subtype selective antagonists in airway tissues from diverse species have shown that the M_3 receptor is the primary subtype responsible for bronchial and tracheal smooth muscle contraction (Roffel *et al*. 1988, Roffel *et al*. 1990). The anticholinergics are primarily used as bronchodilators to reverse the action of vagally derived ACh on airway smooth muscle contraction in the treatment of COPD. As the inhaled antocholinergics have been shown to be associated with a significantly increased risk of cardiovascular death among COPD patients (Singh *et al*. 2008, Macie *et al*. 2008, Lee *et al*. 2008) it is imperative to understand the molecular mechanism of the M_3 receptor antagonist in further detail in conditions of ischaemia-reperfusion injury.

The current study indicates that the M_3 muscarinic receptor antagonist DAU 5884 significantly exacerbates myocardial injury in *ex vivo* conditions of oxidative stress by premature opening of the MPTP. DAU 5884 significantly reduced the depolarisation and hypercontracture time of the isolated cardiac myocytes resulting in cell death. As previously discussed in section 6.4 and also shown in this study (7.3.1) regarding the increased myocardial ischaemia reperfusion injury observed with DAU 5884 treatment, the current study indicates a novel mechanism for DAU 5884 induced cardiotoxicity in stress conditions. We postulate that these observations indicate a central role for the MPTP in DAU 5884 induced toxicity in our models of ischaemia reperfusion injury.

Opening of the MPTP is known to be involved in various pathological conditions including ischaemia reperfusion injury (Yellon and Hausenloy 2007). The MPTP remains closed during ischaemia and has been shown to open during the early minutes of reperfusion when conditions that increase the probability of its opening prevail, such as high mitochondrial calcium, build-up

of reactive oxygen species and inorganic phosphate load (Griffiths and Halestrap 1995, Di Lisa *et al*. 2001). These conditions disrupt the osmotic barrier between the mitochondria and the cytosol, and upon MPTP opening, allow free passage of molecules smaller than 1.5KDa (Halestrap and Pasdois 2009, Yellon and Hausenloy 2007). This initiates a colloidal osmotic pressure on the mitochondrial membrane which leads to cell swelling and eventual rupture of the outer mitochondrial membrane releasing pro-apoptotic factors such as cytochrome c to the inter-membrane space (Halestrap *et al*. 2004). MPTP opening also allows permeability to protons, which results in uncoupling of oxidative phosphorylation and consequently ATP depletion. This in turn can activate degradative enzymes such as phospholipases and proteases and also disrupt ionic and metabolic homeostasis (Halestrap *et al*. 2002). These effects can lead to irreversible cell damage and eventually result in necrotic death.

We have also demonstrated that the MPTP blocker CsA protected the heart from the damage caused by ischaemia reperfusion injury alone in the Langendorff studies. CsA has been previously shown to reduce infarction in ischaemic reperfusion heart models thereby protecting the myocardium (Crompton *et al*. 1988, Griffiths and Halestrap 1993, Shanmuganathan *et al*. 2005). Interestingly, our data demonstrates that the co-administration of CsA with DAU 5884 abrogated the injury inflicted by DAU 5884 alone during ischaemia reperfusion injury. The protective effects of CsA were further confirmed by the findings of the laser induced oxidative stress model. The results showed that CsA alone delayed the depolarisation and hypercontracture time of cardiac myocytes and its co-administration with DAU 5884 also reversed the injury induced by DAU 5884.

Furthermore, as also discussed in section 6.4, the current study also shows that CsA activates pro-survival kinases Akt and ERK1/2 and inhibits stress activated protein, SAPK/JNK, to protect

myocardium against ischaemia reperfusion injury. Phosphorylation of Akt and ERK1/2 is known to be integral to the reperfusion injury salvage kinase pathway that converges on the MPTP (Davidson *et al*. 2006, Hausenloy and Yellon 2007). Various studies have shown that the activation of Akt and ERK 1/2 protects the heart from ischaemia reperfusion injury (Hausenloy *et al*. 2005, Hu *et al*. 2008) and have been discussed in detail in sections 4.4, 5.4 and 6.4.

An apoptotic stimulus causes the pro-apoptotic protein, Bax, to undergo a conformational change and translocate to the mitochondria (Yamaguchi and Wang 2001) inducing release of cytochrome c either by opening of the MPTP or by forming a pore in the outer mitochondrial membrane (Marzo *et al*. 1998). Studies have shown that activation of Akt prevents apoptosis by inhibiting the conformational change required for Bax to translocate to the mitochondria (Tsuruta *et al*. 2002). Akt has also been shown to inhibit release of mitochondrial cytochrome c (Kennedy *et al*. 1999) and as one potential route for its release is via MPTP, it may be postulated that Akt may suppress apoptosis via inhibiting the opening of the MPTP.

Furthermore, the current study also showed that DAU 5884 led to a significantly increase in the stress activated signaling proteins SAPK/JNK. Studies have shown that JNK is activated in response to environmental stresses including heat shock, UV radiation, osmotic shock and inflammatory cytokines (Nishina *et al*. 2004). Evidence in support for activation of SAPK/JNK and its association with apoptosis and cell hypertrophy has been derived from various investigations in *in vitro* and *in vivo* systems. Wang *et al*. (1998) showed that activation of JNK in neonatal rat cardiac myocytes induced characteristic features of hypertrophy and also induced apoptosis. Activation of SAPK/JNK has also been shown to result in apoptotic death of primary neonatal rat cells (Luo *et al*. 1998). Furthermore, myocardial ischaemia-reperfusion has also been shown to activate JNK resulting in apoptosis (Yin *et al*. 1997). Activation of JNK has been

shown by various studies to promote apoptosis. Luo *et al*. (1998) showed that dopamine activates the JNK pathway, including increases in JNK activity, phosphorylation of c-jun and subsequent increases in c-jun protein levels to induce apoptosis in a time and concentration-dependent manner in primary neonatal rat cells. The activation of JNK preceded apoptosis and was persistently sustained during the process of apoptosis. Using a JNK inhibitor prevented both dopamine-induced JNK activation and apoptosis.

Our results also showed that DAU 5884 led to inhibition of the levels of the cell survival signaling proteins Akt and ERK 1/2. Inhibition of Akt and ERK 1/2 has been well documented to inhibit cell survival and promote apoptosis. Granado-Serrano *et al*. (2006) showed that quercetin, a common flavonoid induced apoptosis by direct activation of the caspase cascade and inhibiting cell survival proteins Akt and ERK 1/2 in human hepatoma HepG2 cells. Ostrakhovitch and colleagues (2005) showed that the MEK/ERK pathway plays an important role in down-regulation of p53 and cell survival, and that inhibition of ERK 1/2 can lead to apoptosis via nuclear relocation of apoptosis. Krystal *et al*. (2002) also demonstrated that inhibition of Akt inhibited cell growth and promoted apoptosis in small cell lung cancer cells.

Taken together, this study provides the first evidence that DAU 5884 induced cardiotoxicity is due to the opening of the MPTP in the setting of ischaemia reperfusion injury. The collective findings of the previous chapter and the present study has provided a detailed understanding of the molecular mechanism involved in the M_3 mAChR antagonist induced injury to the myocardium. We have also shown that CsA not only protected the myocardium from the ischaemia reperfusion injury but its co-administration with DAU 5884 also prevented the damage mediated by DAU 5884 alone.

Chapter Eight: General Discussion

8.1 Summary of findings

The non-selective M_1-M_3 antagonist, ipratropium bromide is widely used in the management and treatment of pulmonary conditions such as COPD (Restrepo 2007) but the long-term use of such anticholinergics has been shown to increase the risk of cardiovascular death, myocardial infarction or stroke in COPD patients with underlying CVDs raising concerns over the safety profile of these anticholinergics (Singh *et al*. 2008, Ogale *et al*. 2010, Macie *et al*. 2008). As COPD is also responsible for other systemic pathologies, including underlying CVD (Macnee *et al*. 2008, Maclay *et al*. 2007), it is therefore imperative to ascertain the effect of such anticholinergics and the activity of specific anticholinergics in the setting of myocardial ischaemia reperfusion.

The overall aim of this project was to investigate and understand the effects of individual mAChR antagonists on the myocardium in the setting of myocardial ischaemia reperfusion and on the cardiac myocytes under conditions of oxidative stress. In addition, we also investigated the signalling pathway of individual mAChRs antagonists under the conditions of oxidative stress.

Our results showed that ipratropium bromide, a non-selective M_1-M_3 mAChR antagonist significantly increased the infarct size of the Langedorff perfused heart in the setting of myocardial ischaemia reperfusion but not under normoxic conditions. Ipratropium bromide also decreased the cell viability of cardiac myocytes as revealed by a decrease in MTT reductase activity. Harvey *et al*. (2014) explored the effects of ipratropium bromide on myocardial injury in non-clinical models of simulated myocardial ischaemia reperfusion injury. Furthermore, that

study also evaluated the involvement of apoptosis and necrosis via flow cytometry. The results showed that ipratropium led to a significantly increased infarct size in the isolated perfused rat heart and to decreased cell viability in rat ventricular cardiac myocytes, as shown by a decrease in MTT reductase activity, in a dose-dependent manner. The results also showed that the administration of ipratropium bromide at the onset of re-oxygenation resulted in an increase in both apoptotic and necrotic myocyte death. Loss of cardiac myocyte during ischaemia reperfusion injury via apoptosis and necrosis has also previously been revealed (Kung *et al*. 2011).

Harvey *et al*. (2014) also showed that administration of ipratropium at the onset of re-oxygenation led to increased levels of cleaved caspase-3 in ventricular myocytes following hypoxia and re-oxygenation. Caspase-3 is an executioner caspase in the caspase-dependent apoptotic signalling cascade (Riedl and Shi 2004). It exists as an inactive pro-caspase dimer or zymogen with zero activity and is activated by the initiator caspases such as caspase-8 and caspase-9 (McIlwain *et al*. 2015). In addition, Harvey *et al*. (2014) also showed that the ipratropium induced myocardial injury was abrogated when used in conjunction with an irreversible inhibitor of caspase-3 activation, Z-DEVD-FMK. The study thereby indicated that ipratropium bromide exacerbated myocardial ischaemia reperfusion injury via apoptotic and necrotic associated pathways. Our findings confirm that the non-selective mAChR antagonist ipratropium bromide exacerbates myocardial injury following ischaemia reperfusion injury in rat hearts and reduces the cell viability of isolated cardiac myocytes via decrease in MTT reductase activity in a dose-dependent manner.

The M_2 mAChR is the predominant subtype present in the mammalian heart but studies have shown that M_1 and M_3 also exist (Hulme *et al*. 1990, Caulfiled 1993). As ipratropium bromide is a non-selective mAChR antagonist, we aimed to investigate the role of individual mAChR antagonists in the setting of myocardial ischaemia reperfusion injury. We investigated the effects of the M_1 mAChR antagonist telenzepine dihydrochloride in the setting of myocardial ischaemia reperfusion injury and oxidative stress. The results showed that telenzepine dihydrochloride had no significant effect on the heart nor on the cardiac myocytes.

We then investigated the effects of the M_2 and M_3 mAChR antagonists AF-DX 116 and DAU 5884, respectively, in the setting of myocardial ischaemia reperfusion injury and oxidative stress. Our results did reveal the detrimental effects of administering the M_2 mAChR antagonist AF-DX 116, and the M_3 mAChR antagonist DAU 5884, in the setting of simulated myocardial ischaemia reperfusion and in conditions of oxidative stress. This study showed that administration of these antagonists increased the infarct size in the setting of ischaemia reperfusion injury in a dose dependent manner. AF-DX 116 and DAU 5884 also caused a concentration-dependent decrease in the viability of cardiac myocytes as revealed by a decrease in MTT reductase activity. Furthermore, treatment with AF-DX 116 and DAU 5884 led to a reduction in the time taken to depolarisation and hypercontracture of cardiac myocytes using the model of laser induced oxidative stress.

Mitochondrial dysfunction has been shown to be an underlying cause of ischaemia reperfusion injury (Crompton *et al*. 1999). Opening of the MPTP is known to be involved in various pathological conditions including ischaemia reperfusion injury (Yellon and Hausenloy 2007). Using a laser beam to induce MPTP opening is an established method to track the opening of the

MPTP of isolated cardiac myocytes via recording the time taken to depolarisation and hypercontracture (Hausenloy *et al*., 2002, Yellon and Hausenloy, 2007). Gharanei *et al.* (2013) showed that premature opening of the MPTP is involved in mediating lethal permeability changes that initiate cell death caused by drug induced myocardial injury. The results of the current study have shown that treatment with AF-DX 116 and DAU 5884 reduced the time taken to depolarisation and hypercontracture of cardiac myocytes using the model of laser induced oxidative stress. Our findings therefore support the published literature that premature opening of the MPTP is involved in mediating lethal permeability changes to initiate myocardial damage.

Data obtained from western blot analyses showed that both AF-DX 116 and DAU 5884 caused a significant increase in the levels of the stress activated protein SAPK/JNK and also inhibited the pro-survival proteins Akt and ERK 1/2. Activation of stress activated protein kinases including SAPK/JNK in stress conditions and myocardial ischaemia reperfusion injury have been well documented (Yin *et al*. 1997, Nishina *et al*. 2004). Evidence in support for activation of SAPK/JNK and its association with apoptosis and cell hypertrophy has been derived from various investigations in *in vitro* and *in vivo* systems. Wang *et al*. (1998) showed that activation of JNK in neonatal rat cardiac myocytes induced characteristic features of hypertrophy and also induced apoptosis. Activation of SAPK/JNK has also been shown to result in apoptotic death of primary neonatal rat cells (Luo *et al*. 1998). Kim *et al*. (2001) also showed that activation of SAPK/JNK leads to cell death and its inhibition suppresses cell death in human U937 leukaemia cells. Our results, in line with these earlier findings, suggest a key role for SAPK/JNK in the pathophysiology of cardiac injury in response to various stress conditions, including ischaemia reperfusion injury.

Our results have also shown that administration of the natural mAChR agonist, ACh, significantly decreased the infarct size in the setting of myocardial ischaemia reperfusion. In addition, ACh treatment increased the time taken to depolarisation and hypercontracture of cardiac myocytes using the model of oxidative stress.

The cytoprotective properties of ACh have been well documented (Critz *et al*. 2005, Li *et al*. 2011) and the resulting activation of muscarinic receptors has been shown to provide protection against various cellular insults (De Sarno *et al*. 2003). ACh has also been shown to provide protection against myocardial ischaemia reperfusion injury since it led to reduced infarct size in rats (Richard *et al*. 1995, Yang *et al*. 2005). ACh has been previously shown to be released endogenously in rat heart in the absence of neuronal activity (Brown *et al*. 1982). Harvey *et al*. (2014) also used an ACh assay kit to confirm the presence of endogenous levels of ACh *in vitro*. Furthermore, ACh was also shown to reduce caspase-3 levels thereby providing myocardial protection against hypoxia re-oxygenation partly by a reduction in apoptosis (Harvey *et al*. 2014).

Previous studies have shown that CsA treatment protects the myocardium from ischaemia reperfusion injury (Gharanei *et al*. 2013, Hausenloy *et al*. 2003, Griffiths and Halestrap, 1993). Our results confirm previous findings and show that the administration of the MPTP blocker CsA, led to decreased infarct size in rat hearts in the setting of myocardial ischaemia reperfusion injury. CsA treatment also increased the time taken to depolarisation and hypercontracture of cardiac myocytes using the model of oxidative stress. Interestingly, our data demonstrates that CsA also prevents AF-DX 116 and DAU 5884-induced damage to the myocardium during ischaemia reperfusion injury. We therefore postulate that these observations suggest a central

role for the MPTP in AF-DX 116 and DAU 5884-induced toxicity in our models of ischaemia reperfusion injury.

Furthermore, our results also showed that treatment with ACh and CsA led to activation of the pro-survival proteins Akt and ERK 1/2 and to significantly reduced levels of the stress activated proteins SAPK/JNK. The activation of Akt and ERK 1/2 has been shown to mediate protection against various cellular insults including ischaemia reperfusion injury (Matsui *et al*. 1999, Yue *et al*. 2000, Hausenloy and Yellon 2004). The activation of the PI3K-Akt or the MEK- ERK 1/2 has been attributed to inactivate the pro-apoptotic proteins such as BAD and BAX thereby preventing apoptosis (Datta *et al*. 1997, Yamaguchi and Wang 2001). Hausenloy *et al*. (2005) showed that the activation of the RISK pathway comprising of Akt and ERK 1/2 reduces the damage to the myocardium from ischaemia reperfusion injury. Our data therefore supports the literature from previous findings that activation of Akt and ERK reduces myocardial ischaemia reperfusion injury.

As miRNAs have been identified as biomarkers for cardiovascular diseases and myocardial injury (Ai *et al*. 2010), we investigated the miRNA expression profile of M_2 mAChR antagonist AF-DX 116 treatment on hearts undergoing ischaemia reperfusion injury. Our results showed that AF-DX 116 led to significantly decreased expression levels of miR-1 and miR-133b, below the levels of the ischaemia reperfusion control groups. Previous studies have also shown decreased levels of miR-1 and miR-133b in infarcted tissue in patients with myocardial infarction as compared to healthy adult hearts (Bostjancic *et al*. 2010). Our findings therefore suggest that alterations in miR-1 and miR-133b expression play important regulatory roles in AF-DX 116-mediated injury.

8.2 Limitations and Future investigations

Further studies are required to investigate, in an *in vivo* model, whether the M_2 and M_3 mAChRs antagonists AF-DX 116 and DAU 5884 associated cardiotoxicity is still observed. Furthermore, the effects of chronic administration of AF-DX 116 and DAU 5884, alone or with the co-administration of cardioprotective agents such as CsA, warrants further investigation. Chronic treatment in an *in vivo* model differs significantly from an acute *in vitro* model, as a chronic *in vivo* model would provide more accurate findings since it would involve other factors which may counter the toxic effects of AF-DX 116 and DAU 5884. These factors include blood, hormones, neurones, anti-toxins and other organs.

The results from this study also showed that CsA decreased the infarct size in the Langendorff perfused heart and also decreased the time to depolarisation and hypercontracture of cardiac myocytes under oxidative stress. Therefore, the results do provide the evidence for a direct protective effect of CsA on the MPTP. Previous studies have shown that CsA protects the heart from ischaemia reperfusion injury and oxidative stress via inhibition of the MPTP (Hausenloy *et al*. 2002, Hausenloy *et al*. 2003, Halestrap *et al*. 2004). CsA binds to Cyp-D and causes a conformational change in its morphology preventing it from binding to ANT thereby inhibiting MPTP opening (Tanveer *et al*. 1996). Therefore, further studies using specific Cyp-D blockers will provide a more accurate understanding of the drug treatment on the MPTP.

As previously discussed in chapters 5 and 7 our results showed that AF-DX 116 and DAU 5884 significantly reduced the time to depolarisation and hypercontracture for isolated cardiac myocytes. Oxygen derived free radicals and ATP depletion play an important role in the pathophysiology of cardiac myocyte depolarisation and hypercontracture (Bognar *et al.* 2006, Abdallah e*t al.* 2010). We hypothesise that AF-DX 116 and DAU 5884 may be involved in

producing oxygen derived free radicals that may have resulted in premature opening of the MPTP thereby releasing pro-apoptotic factors such as cytochrome c. Therefore, free radical scavengers such as superoxide dismutase and glutathionine peroxidase could be used in further studies to test the involvement of free radicals in AF-DX 116 or DAU 5884 mediated injury. Furthermore, we observed that AF-DX 116 and DAU 5884 significantly increased the levels of SAPK/JNK and inhibited the expression levels of Akt and ERK 1/2. To investigate whether the increase in SAPK/JNK protein levels is a direct effect of AF-DX 116 and DAU 5884, specific inhibitors of SAPK/JNK such as CEP-1347/KT-7515 should be used. Furthermore, our results have shown that CsA significantly increases levels of Akt and ERK 1/2. The activation of Akt and ERK 1/2 at the onset of reperfusion has been shown to reduce myocardial reperfusion injury (Hausenloy *et al*. 2005, Hu *et al*. 2008). Our data therefore supports the literature from previous findings that activation of Akt and ERK reduces myocardial reperfusion injury. However, to ensure that the increase in Akt and ERK 1/2 was a direct effect of CsA, specific inhibitors of Akt and ERK 1/2, such as wortmannin and UO126 should be used.

We have also demonstrated in chapter 4 the changes in the expression levels of certain miRNAs (miR-1, miR-27a, miR-133a, and miR-133b) in response to treatment with the M_2 mAChR antagonist AF-DX 116. Our results showed that AF-DX 116 caused a significant reduction in the expression levels of miR-1 and miR-133b. However, the precise role of these miRNAs in the cardiotoxicity of AF-DX 116 is not known. Further studies using specific miRNA inhibitors or genetic overexpression can provide a better understanding of their involvement in cardiac injury. In addition, due to lack of time and limited availability of *mir*Vana™ miRNA Isolation kit, only one concentration of AF-DX 116 (1µM) was used. A variety of concentrations of AF-DX 116, ACh and CsA, and combination of AF-DX 116 with CsA, would provide more in-depth findings.

8.3 Conclusion

To conclude, this is the first study to demonstrate the cardiotoxic effects AF-DX 116 and DAU 5884 in the setting of myocardial ischaemia reperfusion and oxidative stress. AF-DX 116 and DAU 5884 cause an increase in the expression of stress activated protein SAPK/JNK, and inhibit the cell survival proteins Akt and ERK 1/2. Moreover, the cardio-toxic effects of these drugs can be prevented when co-administered with CsA or the natural mAChR agonist, ACh which inhibit the SAPK/JNK and activate Akt and ERK 1/2.

References

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